BIOACTIVE 4-PHENYLCOUMARINS FROM *MESUA ELEGANS* AND *MESUA KUNSTLERI*

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FACULTY OF SCIENCE
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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS
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ABSTRACT

Phytochemical analyses on the bark of two Clusiaceae plants, *Mesua elegans* and *Mesua kunstleri*, were carried out. Twenty-four compounds were successfully isolated using several chromatographic techniques; column chromatography (CC), thin layer chromatography (TLC), preparative thin layer chromatography (PTLC), centrifugal partition chromatography (CPC) and high performance liquid chromatography (HPLC). The structures of the isolated compounds were elucidated using various spectroscopic methods; 1D-NMR (^{1}H, ^{13}C and DEPT), 2D-NMR (COSY, HSQC, HMBC and NOESY), UV, IR, HRESIMS and by comparison with data from the literature.

A total of twenty-two compounds were isolated from *M. elegans*, namely mammea A/BA **28**, 5,7-dihydroxy-8-(2-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one **47**, 5,7-dihydroxy-8-(3-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one **48**, mammea A/BB **55**, mammea A/BA cyclo D **187**, mesuagenin A **189**, mesuagenin B **190**, mesuagenin C **191**, ochrocarpin E **192**, mammea A/BB cyclo F **193**, mammea A/BA cyclo F **194**, mesuagenin F **195**, mesuagenin D **196**, isodisparfuran **197**, mesuagenin G **198**, mesuagenin H **199**, mesuagenin I **200**, mesuagenin J **201**, mesuagenin K **202**, mesuagenin L **203**, mesuagenin M **204** and mesuagenin N **205**, of which thirteen are new; mesuagenin A **189**, mesuagenin B **190**, mesuagenin C **191**, mesuagenin F **195**, mesuagenin D **196**, mesuagenin G **198**, mesuagenin H **199**, mesuagenin I **200**, mesuagenin J **201**, mesuagenin K **202**, mesuagenin L **203**, mesuagenin M **204** and mesuagenin N **205**.

From *M. kunstleri*, a total of ten compounds were isolated, which are mammea A/BA **28**, 5,7-dihydroxy-8-(2-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one **47**, 5,7-dihydroxy-8-(3-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one **48**, mammea A/BB **55**, mammea A/BB cyclo D **186**, mammea A/BA cyclo D **187**, mesuagenin E **188**, mesuagenin A **189**, mesuagenin B **190** and mesuagenin C **191**, of which one is new; mesuagenin E **188**.

Bioassay-guided study of the bark from *M. elegans* afforded mesuagenin B **190** from the hexane fraction as the active constituent for acetylcholinesterase (AChE) inhibition activity with an IC_{50} value of 0.70 µM. Further isolation work carried out on this hexane extract afforded more natural 4-phenylcoumarins Semi-synthetic work was done on the major 4-phenylcoumarins from this extract, which afforded seventeen semi-synthetic compounds. All of the natural and semi-synthetic 4-phenylcoumarin analogues were subjected to a SAR study for AChE activity. Another bioassay-guided study was carried out to isolate and evaluate the neuroprotective compounds from the hexane extract of the bark of *M. kunstleri*. Mesuagenin C **191** was isolated as the most active compound; cell viability data demonstrated that this compound significantly increased cell viability (78.99 ± 3.49%) after NG108-15 cells were exposed to 2 mM H_2O_2 and 2 hours of pretreatment with mesuagenin C **191**.

ABSTRAK

Kajian fitokimia telah dijalankan dengan menggunakan bahagian batang daripada dua pokok Clusiaceae, *Mesua elegans* dan *Mesua kunstleri*. Dua puluh empat sebatian telah berjaya dipisahkan dengan menggunakan beberapa kaedah spektroskopi; kromatografi turus, kromatografi lapisan nipis, kromatografi lapisan nipis persediaan, kromatografi partisi empar dan kromatografi cecair prestasi tinggi. Struktur sebatian-sebatian yang dipisahkan telah diilusidasi dengan menggunakan pelbagai kaedah spektroskopi; 1D NMR (^{1}H, ^{13}C dan DEPT), 2D NMR (COSY, HSQC, HMBC dan NOESY), UV, IR, HRESIMS dan perbandingan dengan data-data daripada kesusasteraan.

Sejumlah dua puluh dua sebatian telah dipisahkan daripada *M. elegans*, iaitu mammea A/BA **28**, 5,7-dihidroksi-8-(2-metilbutanoil)-6-[(*E*)-3,7-dimetilokta-2,6-dienil]-4-fenil-2*H*-kromen-2-one **47**, 5,7-dihidroksi-8-(3-metilbutanoil)-6-[(*E*)-3,7-dimetilokta-2,6-dienil]-4-fenil-2*H*-kromen-2-on **48**, mammea A/BB **55**, mammea A/BA siklo D **187**, mesuagenin A **189**, mesuagenin B **190**, mesuagenin C **191**, ochrocarpin E **192**, mammea A/BB siklo F **193**, mammea A/BA siklo F **194**, mesuagenin F **195**, mesuagenin D **196**, isodisparfuran **197**, mesuagenin G **198**, mesuagenin H **199**, mesuagenin I **200**, mesuagenin J **201**, mesuagenin K **202**, mesuagenin L **203**, mesuagenin M **204** dan mesuagenin N **205**, di mana tiga belas daripadanya ialah sebatian baru; mesuagenin A **189**, mesuagenin B **190**, mesuagenin C **191**, mesuagenin F **195**, mesuagenin D **196**, mesuagenin G **198**, mesuagenin H **199**, mesuagenin I **200**, mesuagenin J **201**, mesuagenin K **202**, mesuagenin L **203**, mesuagenin M **204** dan mesuagenin N **205**.

Daripada *M. kunstleri*, sejumlah sepuluh sebatian telah dipisahkan, iaitu mammea A/BA **28**, 5,7-dihidroksi-8-(2-metilbutanoil)-6-[(*E*)-3,7-dimetilokta-2,6-dienil]-4-fenil-2*H*-kromen-2-one **47**, 5,7-dihidroksi-8-(3-metilbutanoil)-6-[(*E*)-3,7-dimetilokta-2,6-dienil]-4-fenil-2*H*-kromen-2-on **48**, mammea A/BB **55**, mammea A/BB siklo D **186**, mammea A/BA siklo D **187**, mesuagenin E **188**, mesuagenin A **189**, mesuagenin B **190** dan mesuagenin C **191**, dimana satu sebatian ialah baru; mesuagenin E **188**.

Kajian berpandukan bioesei dijalankan dengan menggunakan bahagian batang daripada *M. elegans* memberikan mesuagenin B **190** sebagai sebatian yang aktif daripada ekstrak heksana untuk aktiviti penyekatan asetil kolinesterina (AChE) dengan IC_{50} 0.70µM. Kerja pengasingan selanjutnya yang dijalankan dengan ekstrak heksana ini memberikan lebih banyak 4-fenilkomarin semulajadi dan kerja-kerja separa-sintesis dijalankan ke atas sebatian 4-fenilkomarin utama (major) daripada ekstrak ini, dimana tujuh belas sebatian separa-sintesis telah diperolehi. Satu kajian SAR telah dijalankan ke atas semua analog 4-fenilkomarin semulajadi dan separa-sintesis. Satu lagi kajian berpandukan bioesei telah dijalankan untuk mengasing dan menilai sebatian-sebatian pelindung neuro dengan menggunakan ekstrak heksana bahagian batang daripada *M. kunstleri*. Mesuagenin C **191** telah diasingkan sebagai sebatian yang paling aktif; data daya maju sel menunjukkan sebatian ini telah meningkatkan daya maju sel dengan ketara (78.99 ± 3.49%) selepas sel-sel NG108-15 telah didedahkan kepada 2 mM H_2O_2 dan 2 jam prarawatan dengan mesuagenin C **191**.

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ABBREVIATIONS

α	Alfa
β	Beta
$CDCl_3$	Deuterated chloroform
CH_3	Methyl group
cm^{-1}	Per centimetre
COSY	H-H correlation spectroscopy
°C	Degree celcius
^{13}C	13-Carbon
δ	Chemical shift
2D NMR	Two dimensional NMR
DEPT	Distortionless Enhancement by Polarisation Transfer
d	Doublet
dd	Doublet of doublet
FT-NMR	Fourier transform NMR
^1H	Proton
γ	Gamma
g	Gram
HRESIMS	High Resolution Electron Spray Ionization Mass Spectroscopy
HMBC	Heteronuclear Multiple Bond Coherance
HMQC	Heteronuclear Multiple Quantum Coherance
Hz	Hertz
IC_{50}	Concentration required to inhibit 50% of activity
IR	Infrared
J	Coupling constant (Hz)
λ	Lambda (maximum wavelength)
m	Metre

m	Multiplet
MHz	Mega Hertz
mL	Mili litre
MS	Mass spectroscopy
m/z	Mass/charge
μg/mL	Microgram per mili litre
nm	Nanometre
NMR	Nuclear Magnetic Resonance
OMe	Methoxyl group
OH	Hydroxyl group
ppm	Parts per million
R_f	Retention factor
s	Singlet
t	Triplet
μL	Micro litre
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Throughout the ages, humans have depended on nature for their basic needs, including medicines for the treatment of a wide spectrum of diseases[1]. Plants have formed the basis of sophisticated traditional medicine systems, with the earliest records of natural products depicted on clay tablets in cuneiform from Mesopotamia (2600 B.C.). These documented oils from *Cupressus sempervirens* (Cypress) and *Commiphora* species (myrrh), which are still used today to treat coughs, colds and inflammation. The use of natural products as medicines has been described throughout history in the form of traditional medicines, remedies, potions and oils, with many of these bioactive natural products still being unidentified[2]. A vast majority of people on this planet still rely on their traditional *material medica* (medicinal plants and other materials) for their everyday healthcare needs, and their use by different cultures, has been extensively documented[3]. The World Health Organization (WHO) estimated in 1985, that approximately 65% of the population of the world predominantly relied on plant-derived traditional medicines for their primary health care[4]. Interestingly, more than 90% of current therapeutic classes derive from a natural product prototype[4].

Natural products have been the most successful source of potential drug leads[1,2,3,5] because they possess a vast range of unique structures with unusual functional groups that have been an inspiration for creating new entities through synthetic chemistry[6,7,8,9,10]. Their magnificent structural diversity and complexity has urged synthetic chemists to produce them in the laboratory, often with beneficial therapeutic applications in mind. Natural products have been precious as tools for deciphering the logic of biosynthesis, and as platforms for developing front-line drugs, and many drugs

used today are natural products or natural-product derivatives[5, 11]. Indeed, the plant kingdom has proven to be among the best source of natural products, with leading examples such as morphine **1**, vincristine **2**, vinblastine **3**, artemisinin **4**, quinine **5** and paclitaxel **8**, while etoposide **6** and teniposide **7** are examples of potent natural-product derivatives[12].

Amongst all types of forest, the tropical rainforest is considered as the most diverse and dense. There are only three areas in the world where tropical rainforests are found – tropical South America, Central Africa and Southeast Asia. The rainforests of Southeast Asia are believed to be the oldest, and among the most biologically diverse, in the world[13,14,15]. The species richness and endemism of Malaysia is of major global importance. It ranks 21st among all countries in absolute diversity and 20th when adjusted to the country's area. Malaysia has more than 15,000 higher plant species, of which 2,700 species are endemic[16]. Therefore, the Malaysian flora is a potential producer of potent bioactive compounds which could serve as leads in drug discovery research.

Some examples of unique bioactive compounds from this extremely rich flora are rhazinilam **9**, calanolide A **10**, and kingianin A **11**. Rhazinilam **9**, a substance first isolated from *Rhazya stricta* (Apocynaceae), has been re-isolated from a Malaysian plant, *Kopsia singapurensis*, and is an antitubulin alkaloid which possesses both the effect of the vinblastine and taxol classes of compounds and is widely studied for its' activity and synthesis[17,18,19]. In another study, calanolide A **10**, discovered in the extracts from the Malaysian tropical rainforest tree, *Calophyllum lanigerum* (Clusiaceae), is a novel inhibitor of the human immunodeficiency virus (HIV) type 1, and various studies have been carried out on this compound since its' discovery in

1992[20,21,22,23]. Kingianin A **11**, from a Malaysian *Endiandra kingiana* (Lauraceae), is a new polyketide that has an unprecedented pentacyclic carbon skeleton and constitutes the first member of a new chemical series. The (-)-enantiomer of kingianin A **11** posseses a binding affinity evaluated on Bcl-xL by competition against the fluorescent-tagged BH3 domain of the protein Bak[24,25].

Due to the vastness and diversity of this rich flora, many of Malaysian plants have never been the subject of any chemical or biological studies. Therefore, under the framework of French-Malaysian collaboration program, a survey on extracts inhibiting acetylcholinesterase (AChE) was conducted on plants from the Malaysian flora, and it was found that the ethyl acetate extracts from *Mesua elegans* (King) Kosterm. and *Mesua kunstleri* (King) Kosterm. exhibited strong AChE inhibitory activity, with 90% inhibition at 10 μg/mL, for both plants. In addition to the AChE activity, a bioassay-guided study of neuroprotective inhibition activity was also done, since both the activities (AChE inhibition and neuroprotective inhibition activities), are related to neurodegenerative diseases, such as Alzheimer's and Parkinson's disease. Henceforth, a bioassay-guided study and structure-activity relationship (SAR) study of AChE inhibitors from *Mesua elegans* (King) Kosterm. and a bioassay-guided study of neuroprotective inhibition activity from *Mesua kunstleri* (King) Kosterm. shall be reported. Both plants belong to the family Clusiaceae. The plant sample of *M. elegans* was collected from Sintok Forest Reserve, Kedah, while *M. kunstleri* was collected from Rimba Teloi Forest Reserve, Kedah.

The objectives of this research are:

i. to isolate the chemical constituents from *M. elegans* and *M. kunstleri* by using different chromatographic techniques; CC, PTLC, HPTLC, CPC and HPLC.

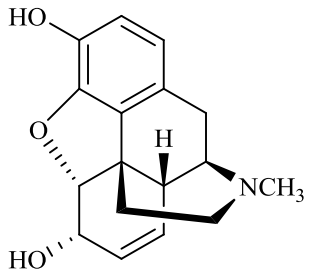
ii. to characterize the chemical structures of the isolated compounds by using various 1D and 2D spectroscopic methods; NMR, UV, IR and LC-MS.

iii. to evaluate the acetylcholinesterase (AChE) inhibitory activity of the isolated compounds from *M. elegans* through bioassay-guided approach and identify the compound/s which is/are responsible as AChE inhibitor/s.

iv. to perform chemical modifications on the isolated major chemical constituents from *M. elegans* and subject the modified compounds for AChE inhibitory activity evaluation.

v. to conduct a structure-activity relationship (SAR) studies on the constituents and chemically modified compounds from *M. elegans* for AChE inhibitory activity.

vi. to evaluate the neuroprotective effects of the isolated compounds from *M. kunstleri* through bioassay-guided method and identify the compound/s which is/are responsible as neuroprotective agent(s).



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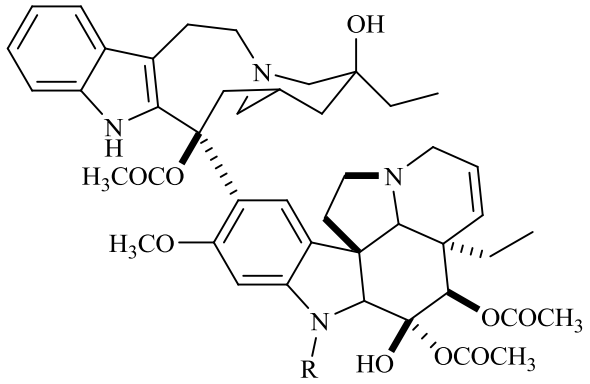

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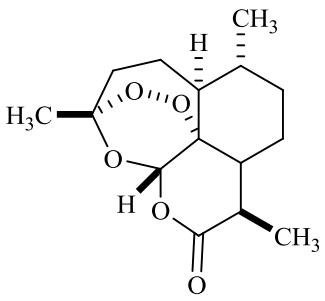

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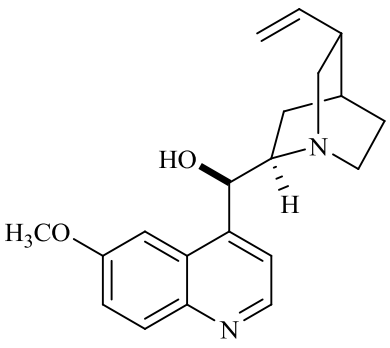

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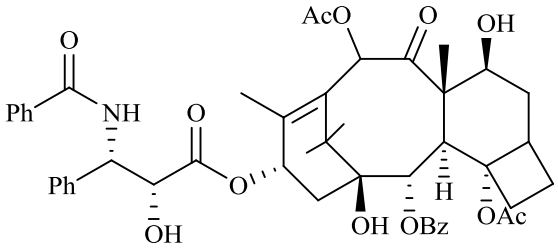

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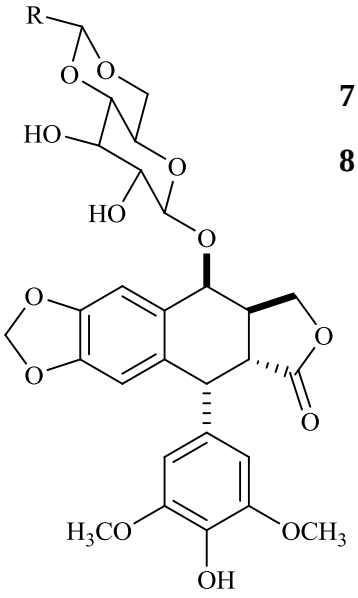

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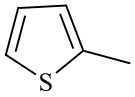
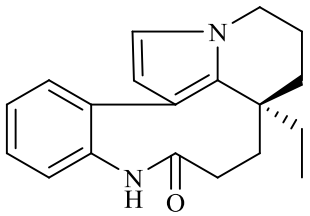
8 R = 



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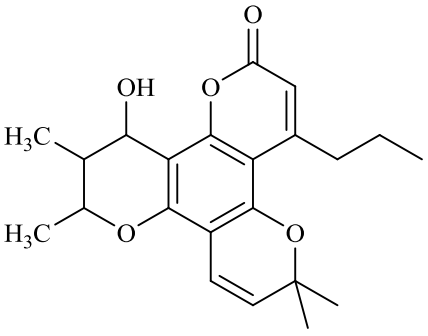

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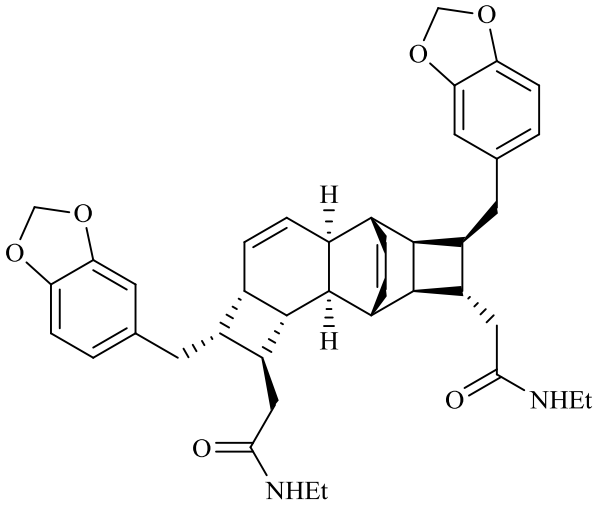

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1.1 Clusiaceae: Distribution and Habitat

The Clusiaceae in Peninsular Malaysia, which was formerly known as the Guttiferae, includes some important and well-known trees. Many plants from this family are known for their timber value and there are extensive stands in parts of the hill forests which are likely to become important. This family is mainly distributed in the tropical regions, comprising 40 genera and more than 1000 species, which are least abundant in Africa. Peninsular Malaysia elaborates 4 genera with 121 species. They can be found in all kinds of habitat, and are an important component of the Peninsular Malaysian Rain Forest. The family includes *Calophyllum, Garcinia, Mammea* and *Mesua*[26].

Calophyllum, Garcinia and *Mesua* are all found from the sea level to the top of the highest mountains; the latter two genera are found mainly in dry land forests, and only *Calophyllum* is common in swamp forests. The genus *Mammea* is rare and is restricted to the lowlands. Several *Mesua* characterise poor sites in ridge forests[27]. Clusiaceae is a family of the main canopy of the forest and no species grows to become an emergent tree[26].

1.2 General Appearance and Morphology

This tropical family is often related with the St. John's Wort-family (Hypericaceae)[28]. Plants from Clusiaceae are usually rather slender, small to medium or big trees, and are occasionally shrubs. Inner bark is usually sticky exudate, which is occasionally very slowly appearing, clear or opaque, white or yellow. The wood is usually hard, dark and dense[26].

Twigs are usually square at first. The leaves are opposite, decussate, coriaceous and shortly stalked; commonly leathery, with oil glands or passages, which is sometimes

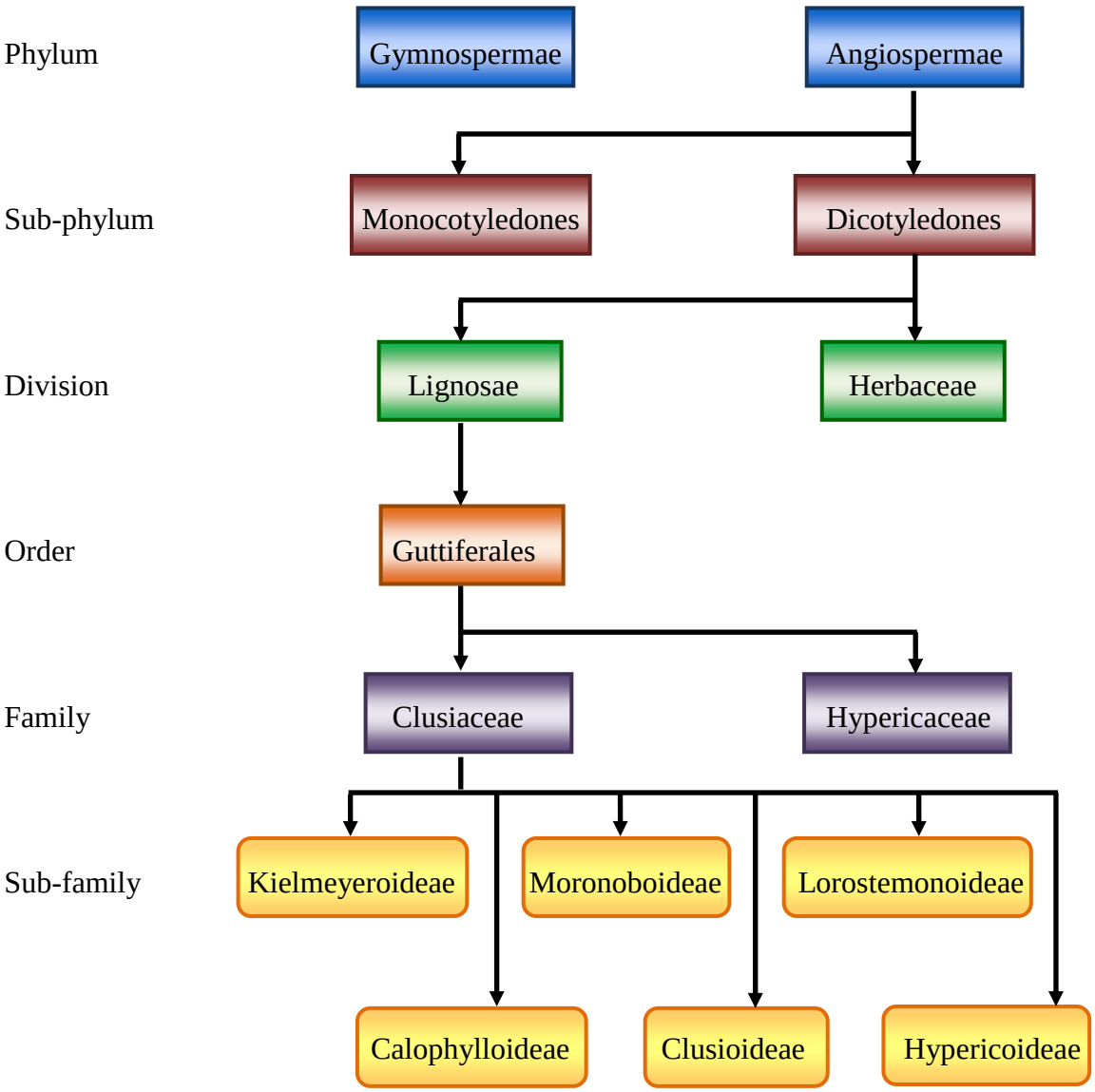
visible as translucent dots[27]. The secondary nerves are usually numerous, more or less straight and parallel, frequently with equally prominent, shorter and intermediate nerves (intercostals); sometimes with tiny reduced needle-like leaves resembling stipules[26].

Flowers are often bright and showy, scented and insect-pollinated; solitary or in short cymose to umbellate groups, terminal, axillary, or on the twigs behind the leaves[28]. It is often with bracteoles below the calyx, with sepals free and usually overlapping (fused at first in *Mammea*) and with petals free and overlapping. The stamens are usually numerous, often variously united as a ring or into bundles and with ovary superior, 1-3-5-(12) chambers, each with 1-2 basal or axile ovules. They are with 1 style and often a big stigma; unisexual and monoecious, dioecious or hermaphrodite[26].

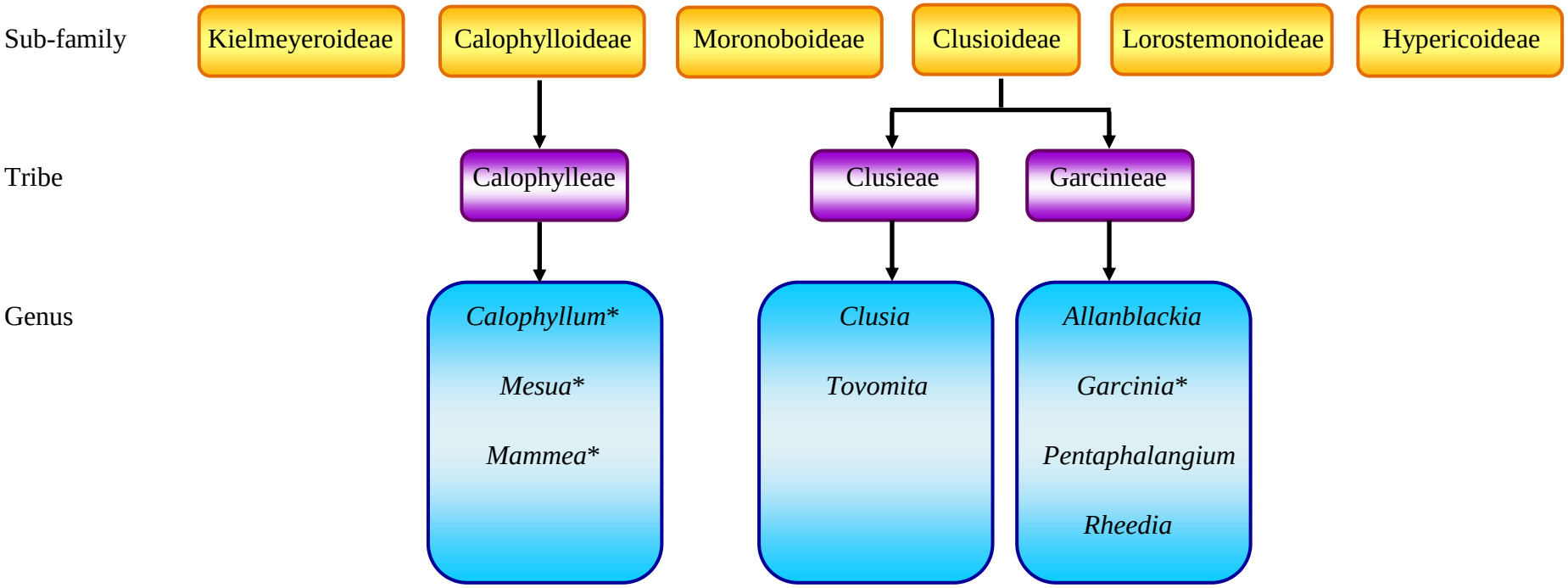
The fruit is a drupe (*Calophyllum* and *Mammea*), a nut (*Mesua*) or a berry with the seeds embedded in pulp (*Garcinia*); sometimes seated on the persistent sepals. The seeds are without endosperm, either with big cotyledons (*Calophyllum*, *Mammea* and *Mesua*) or apparently without cotyledons and germinating with the root and shoot emerging at opposite ends (*Garcinia*)[26].

1.3 Classification of the Clusiaceae[29,30]

Classification of the family Clusiaceae is not an easy task. Determination of a genus is dependent on a combination of characters, for instance those of the petal and fruit. This family is divided into six sub-families: Kielmeyeroideae, Calophylloideae, Clusioideae, Moronoboideae, Lorostemonoideae and Hypericoideae (Scheme 1.1). These sub-families are further divided into a few tribes and each tribe comprises of several genera (Scheme 1.2).



Scheme 1.1: Classification of the Clusiaceae



Scheme 1.2: Genera of the Clusiaceae

* Genus found in Peninsular Malaysia[3]

1.4 The Genus: *Mesua*

The genus *Mesua* is locally known as 'penaga', comprising small to medium trees with very hard and heavy wood. *Mesua ferrea* is the commonest wild species in this genus, and is also a common ornamental[26]. Table 1.1 below lists some of the species of *Mesua* and their distributions.

Table 1.1: Species of *Mesua* and Their Distribution[26,27]

***Mesua* Species**	**Distribution**
M. ferrea Linn.	Malacca, Negeri Sembilan, Pahang, Selangor, Perak, Penang, India, Cambodia and Siam
M. kochummeniana Whitmore	Johor and Pahang (Common in Gunung Lesong Forest Reserve)
M. daphnifolia (Ridley) Kosterm.	Perak and Fraser's Hill, Pahang
M. racemosa (Planch. ex Triana et Planch) Kosterm.	Kelantan, Terengganu, Kedah, Perak, Penang, Pahang, Selangor and Malacca
M. kunstleri (King) Kosterm.	Malacca and central Pahang
M. rosea (Ridley) Kosterm.	Johor
M. elegans (King) Kosterm.	Perak, Selangor, Kedah, Pahang, Negeri Sembilan, Johor and Singapore
M. purseglovei Whitmore	Pahang
M. wrayi (King) Kosterm.	Gunung Korbu and Cameron Highlands
M. lepidota T. Anders.	Terengganu, Pahang, Perak, Selangor, Negeri Sembilan, Malacca, Johor and Sumatra
M. nuda Kosterm. Ex Whitmore	Kelantan, Terengganu, Kedah, Penang, Perak, Pahang (common in Baloh Forest Reserve), Selangor (common in Ulu Gombak Forest Reserve), Malacca, Negeri Sembilan and Johor
M. grandis (King) Kosterm.	Terengganu, Perak and Pahang
M. aff. *assamica* (King et Prain) Kosterm.	Kedah, West Kelantan, Terengganu, Pahang (including Pulau Tioman), Negeri Sembilan, Johor and Assam

1.5 *Mesua*: General Morphology and Appearance

Plants from the genus *Mesua* are bushes or small to large trees, and their crown is monopodial, but becoming sympodial. The branches and foliage sprays are often pendulous. The bole is commonly fluted, especially at butt, sometimes of rather poor form. The bark is smooth to adherent-scaly, sometimes somewhat dippled. The inner bark is firmly fibrous, usually pink to red, with a little, usually clear, sticky exudate appearing as a thin, varnish-like film, or as separate drops. The outer bark is sometimes with an ochre to buff layer inwards. The wood is exceedingly hard, dark inwards, sometimes tangentially banded pale and dark[26].

Leaves are opposite and stiffly coriaceous[27]. They are also glabrous or occasionally glaucous (*M. ferrea* and *M. kochummeniana*), and often shiny. The secondary nerves are usually arising nearly normal to midrib, running parallel nearly to margin then looping; frequently with equally prominent intercostals. It has small or tiny reduced interpetiolar leaves (hypsophylls), sometimes persistent, with paired needle-like stipules[26].

The flowers are bisexual, axillary or terminal, and usually they are stalked with small paired bracts; solitary or cymosely in 1-9 flowered open panicles[27]. They have sepals with 2+2 orientation, decussate, and usually they are round. The petals are usually white with numerous free stamens. The ovaries are with 1 or 2 chambers, each with 1-2 axile ovules[26]. Fruits are usually thinly woody and often not splitting open, and they are usually tipped by the protruding, persistent style base[27]. The fruits often exude resinous droplets, embraced by the persistent sepals (except *M. nuda*), or enveloped by the variously woody to leathery sepals, and often contain 1 to 4 dry seeds[28].

1.6 Biological Importance of *Mesua* species

M. ferrea, known with several common names, such as 'Ironwood', 'Nagkesar' or 'Cobra's saffron', is the most widely studied plant in this genus, and is a medium to large sized well-known tropical tree[31,32]. It is traditionally used as antiseptic, antiasthmatic and antiallergic activities. It is an ingredient of Ayurvedic formulations such as Brahma Rasayana and Chyavanprash which are used to improve immunity[32,33]. It is also used in folk medicine for the treatment of fever, dyspepsis and renal diseases[34,35,36]. The flowers are said to be astringent, and a stomachic and expectorant, and useful in bleeding piles and dysentery.

Leaves and flowers are used in the treatment of snake bite and scorpion sting[34]. The seeds of *M. ferrea* have been recommended for treating painful and inflammatory conditions like arthritis, wound healing and skin diseases[31]. Different aerial parts of *M. ferrea* are traditionally used in the preparation of cosmetics and unguents. The extracts of *M. ferrea* had been studied for their antioxidant and hepatoprotective activity[37,38]. The plant had also been reported to possess antidote to scorpion venom[39], CNS depressant[40], anticancer[41] and antibacterial activity[41,42,43,44,45,46].

The ethyl acetate extract from the leaves of *M. racemosa* was selected for phytochemical investigation due to its significant cytotoxic activity against P388 (leukemia cells) and KB cells (epidermal carcinoma of the nasopharynx) by Morel *et al.*[47]; 100% at a concentration of 10 µg/mL against P388, and 83 and 19% for concentrations of 10 and 1 µg/mL, respectively, against KB cells. They have reported that preliminary biological evaluation of racemosol **12** and mammea A/AC cyclo F **13** exhibited a weak cytotoxicity activity against KB cells; 49 and 23% for **12** and 32 and 17% for **13** at 10 and 1 µg/mL, respectively.

Chemical constituents from *M. daphnifolia* were tested *in vitro* for their cytotoxic activities against four cell lines, MDA-MB-231 (human estrogen receptor negative breast cancer), HeLa (cervical carcinoma), CEM-SS (T-lymphoblastic leukemia) and CaOV3 (human ovarian cancer) by Ee *et al*.[48]. Cudraxanthone G **14** showed a broad spectrum of activity against the MDA-MB-231, HeLa and CEM-SS cell lines with the IC_{50} values of 1.3, 4.0 and 6.7 μg/mL, respectively. Besides **14**, the MDA-MB-231 cell line was also found to be very susceptible towards ananixanthone **15** with an IC_{50} value of 4.6 μg/mL. Meanwhile, inhibitory activities were observed for friedelan-1,3-dione **16** against the HeLa cell line with an IC_{50} value of 4.6 μg/mL, and weaker activity was observed for lup-20(29)-en-3β-ol **17** with an IC_{50} value of 8.6 μg/mL. The CEM-SS cell line was found to be moderately susceptible towards **17** with the IC_{50} value of 13.8 μg/mL. A moderate IC_{50} value of 9.0 μg/mL was observed for euxanthone **18** towards the CaOV3 cell line.

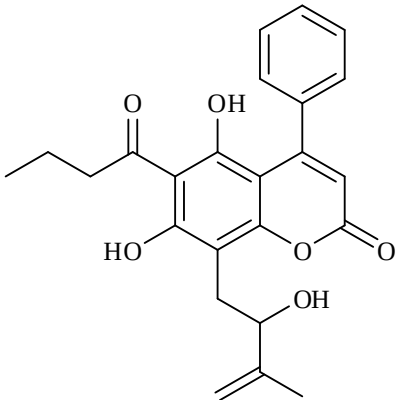
Meanwhile, the same group, Ee *et al*.[49] has performed a study on the hexane extract of the stem bark of *M. corneri*, which was found to be cytotoxic against CEM-SS cell lines with an IC_{50} value of less than 30 μg/mL. Rubraxanthone **19** was isolated from this extract with an IC_{50} value of 5.0 μg/mL.

Congestiflorone **20** from *M. congestiflora* had been subjected to cytotoxic tests against various cancer cell lines by Ee *et al.*[50], including Raji (human B lymphocyte), SNU-1 (human gastric carcinoma), LS-174T (human colorectal adenocarcinoma), HeLa (human cervical cells), SK-MEL-28 (human malignant melanoma cells), NCI-H23 (human lung adenocarcinoma), IMR-32 (human neuroblastoma), Hep-G2 (human hepatocellular liver carcinoma) and K562 (human erythroleukemia cells). Compound **20**

showed weak cytotoxic activities against the entire cancer cells except for Raji which gave cytotoxic activity with an IC_{50} value of 10.85 ± 1.10 μg/mL.

Teh *et al*.[51] carried out *in vitro* cytotoxic activities of the isolates from *M. beccariana* against a panel of human cancer cell lines; Raji, SNU-1, K562, LS-174T, HeLa, SK-MEL-28, NCI-H23, IMR-32 and Hep-G2 using the MTT assay. Mesuadione **21**, beccamarin **22**, betulinic acid **23** and stigmasterol **24** displayed strong inhibition of Raji cell proliferation, while the proliferation rate of HeLa and SK-MEL-28 were strongly inhibited by **22** and **24**, with IC_{50} values of less than 3.91 and 3.90 μg/mL, respectively.

In a study carried out by the same group[52,53], it was found that the methanol extracts of *M. beccariana* and *M. ferrea* showed high antioxidant activities with EC_{50} values of 12.70 and 9.77 μg/mL, respectively, which are comparable to ascorbic acid (EC_{50} = 5.62 μg/mL). The methanol extract of *M. beccariana* showed some activities against Gram-positive bacteria; *Bacillus cereus*, methicillin-sensitive *Staphylococcus aureus* (MSSA), and methicillin-resistant *Staphylococcus aureus* (MRSA), while the hexane extract also contributed some activities towards *Bacillus cereus*.



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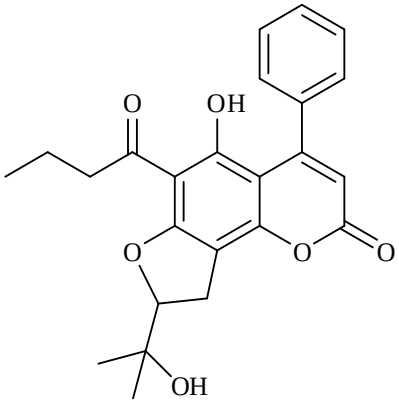

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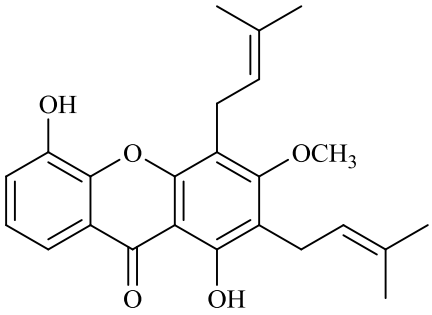

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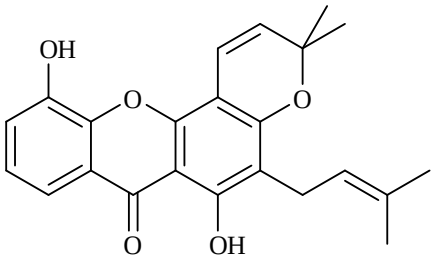

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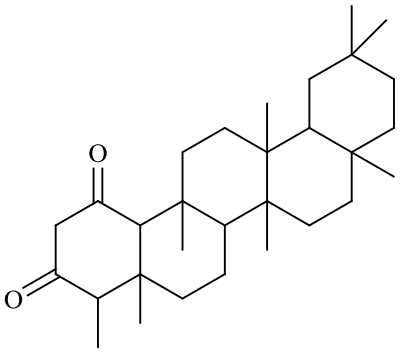

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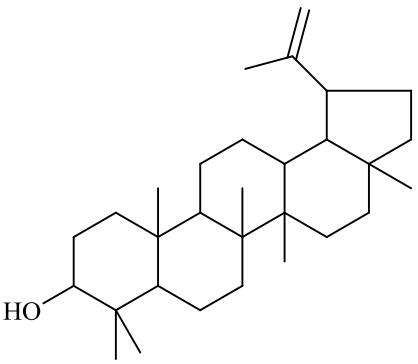

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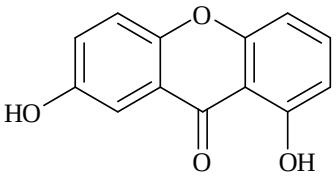

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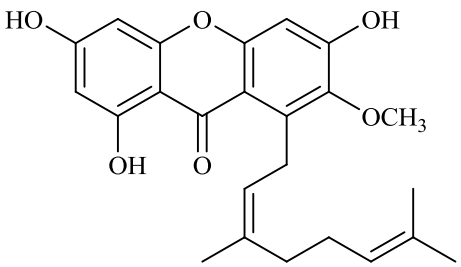

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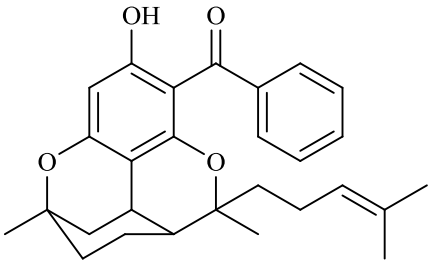

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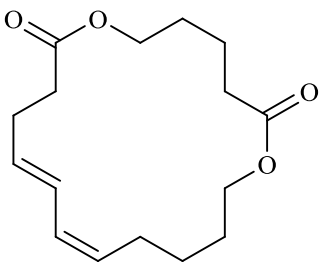

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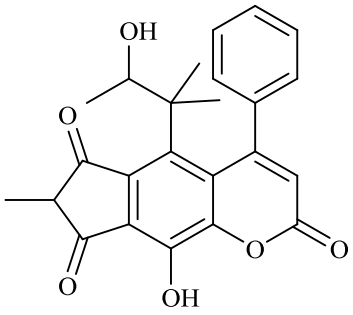

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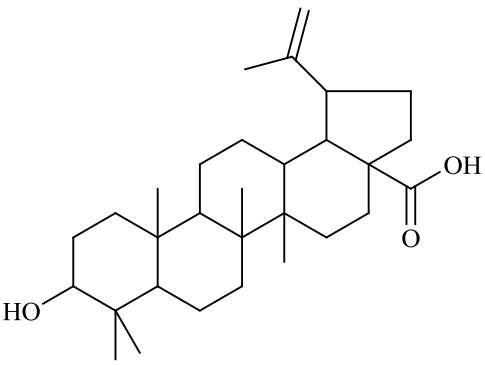

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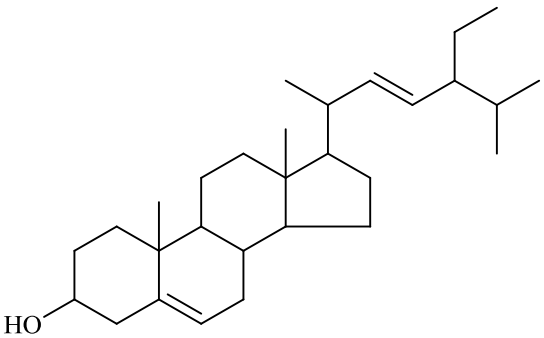

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CHAPTER 2

GENERAL CHEMICAL ASPECTS

Extensive work on the isolation and identification of chemical compounds from *Mesua* species, as well as their biological activities have been reported, as many of the plants from this genus are known for their medicinal value. *Mesua* species are known to furnish various types of compounds, including coumarins, xanthones, flavones, terpenoids and phenolics. Amongst the species from this genus, *Mesua ferrea* is the most widely studied, mainly due to its availability and biological value[8]. Table 2.1 below lists the chemical constituents found in *Mesua* species and their references.

Table 2.1: Chemical Constituents of *Mesua* species

***Mesua* sp.**	**Plant part**	**Compounds**	**References**
Mesua beccariana (Baill.) Kosterm.	Stem bark	Beccamarin **22** Betulinic acid **23** 6-Deoxyjacareubin **25** 2,5-Dihydroxy-1,3,4-trihydroxyanthraquinone **26** Friedelin **27** Mammea A/BA **28** Mesuadione **21** Mesuarianone **29** Mesuasinone **30** 4-Methoxy-1,3,5-trihydroxyanthraquinone **31** Stigmasterol **24**	51,52,53,54,55
Mesua congestiflora	Roots	Congestiflorone **20** α-Mangostin **32**	52,53,50,56

***Mesua* sp.**	**Plant part**	**Compounds**	**References**
Mesua Corneri Linn.	Stem bark	Friedelan-1,3-dione **16** Friedelin **27** Inophyllin B **33** Rubraxanthone **19** Stigmasterol **24**	[49]
Mesua daphnifolia (Ridley) Kosterm.	Stem bark	Ananixanthone **15** Cudraxanthone G **14** Daphnifolin **34** Euxanthone **18** Friedelan-1,3-dione **16** Friedelin **27** Lup-20(29)-en-3β-ol **17**	[48,57,58]
Mesua ferrea Linn.	Bark	(-)-Epicatechin **35** Ferruol A **36** 5-Hydroxy-1-methoxyxanthone **37** Mammea B/BB **38** Mammea C/BB **39** Mesuferrol A **40** Mesuferrol B **41**	[59,60]
	Flower	Assamene **42** 8,9-Dihydro-5-hydroxy-6-(2-methylbutanoyl)-4-phenyl-8-(prop-1-en-2-yl)-furo[2,3-*h*]-chromen-2-one **43** 8,9-Dihydro-5-hydroxy-6-(3-methylbutanoyl)-4-phenyl-8-(prop-1-en-2-yl)-furo[2,3-*h*]-chromen-2-one **44** 5,7-Dihydroxy-6-(2-methylbutanoyl)-8-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one **45** 5,7-Dihydroxy-6-(3-methylbutanoyl)-8-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-	[46]

***Mesua* sp.**	**Plant part**	**Compounds**	**References**
		phenyl-2*H*-chromen-2-one **46** 5,7-Dihydroxy-8-(2-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one (DMDP-1) **47** 5,7-Dihydroxy-8-(3-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one (DMDP-2) **48** 5-Hydroxy-6-isobutyryl-8-methyl-8-(4-methylpent-3-enyl)-4-phenyl-2*H*-pyrano[2,3-*h*]chromen-2-one **49** 5-Hydroxy-8-methyl-6-(2-methylbutanoyl)-8-(4-methylpent-3-enyl)-4-phenyl-2*H*-pyrano[2,3-*h*]chromen-2-one **50** Mammea A/AA cyclo D **51** Mammea A/AA cyclo F **52** Mammea A/AB cyclo F **53** Mammea A/AD cyclo F **54** Mammea A/BA **28** Mammea A/BB or isomammeisin **55** Surangin C **56**	
	Heartwood	1,5-Dihydroxyxanthone **57** Euxanthone **18** Euxanthone-7-methyl ether **58** Ferrxanthone **59** Mesuaxanthone A **60** Mesuaxanthone B **61** β-Sitosterol **62**	61,62,63
	Leaves	Mesuein **63**	64
	Root bark	Betulinic acid **23** Caloxanthone C **64** 1,8-Dihydroxy-3-methoxy-6-methylanthraquinone **65**	52,65

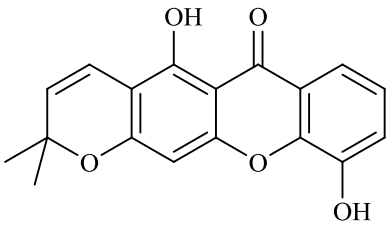
***Mesua* sp.**	**Plant part**	**Compounds**	**References**
		Friedelin **27** Macluraxanthone **66** Mesuaferrin A **67** Mesuaferrin B **68** Mesuaferrin C **69** β-Sitosterol **62** Tovopyrifolin C **70**	
	Seeds	Mammea A/AA or mammeisin **71** Mammea A/AB **72** Mammea A/AB cyclo D or mammeigin **73** Mammea A/AD cyclo D or mesuagin **74** Mesuarin **75** Mesuol **76**	[66,67,68,69]
	Stamens	β-Amyrin **77** Mesuaferrol **78** Mesuaferrone A **79** Mesuaferrone B **80** Mesuanic acid **81** β-Sitosterol **62**	[70,71,72,73]
Mesua ferrea Linn.	Stem bark	1,6-Dihydroxyxanthone **82** (-)-Epicatechin **35** Mesuabixanthone A **83** Mesuabixanthone B **84** Pyranojacareubin **85**	
	Bark, leaves, buds and flowers in full blossom	Acetic acid **86** 4-Acetyl-1-methylcyclohexene **87** 3-Acetyl-3-methylcyclopentene **88** α-Acoradiene **89** Alloaromadendrene **90** Ar-curcumene **91** Aromadendrene **92** *Trans*-α-bergamotene **93** β-Bisabolene **94**	[74]

***Mesua* sp.**	**Plant part**	**Compounds**	**References**
		(*E*)-γ-Bisabolene **95** (*E*)-α-Bisabolene **96** (*Z*)-γ-Bisabolene **97** (*Z*)-α-Bisabolene **98** β-Bourbonene **99** α-Cadinol **100** δ-Cadinene **101** T-cadinol **102** β-Calacorene **103** β-Caryophyllene **104** Caryophyllene oxide **105** 1,8-Cineole **106** Cubenol **107** 1-*Epi*-cubenol **108** α-Copaene **109** α-Cubebene **110** Cyclosativene **111** Cyperene **112** 2-Decanone **113** (*E*)-2-Decenal **114** 1,4-Dimethoxybenzene **115** Drima-7,9(11)-diene **116** Elema-1,3,11(15)-trien-12-al **117** β-Elemene **118** (*E*,*E*)-Farnesol **119** (*E*,*E*)-Farnesyl acetate **120** Geraniol **121** Germacrene D **122** Globulol **123** Hexanal **124** Hexanol **125** (*Z*)-3-Hexen-2-one **126** 5-Hexen-2-one **127**	

***Mesua* sp.**	**Plant part**	**Compounds**	**References**
		(*Z*)-3-Hexenyl acetate **128** (*Z*)-3-Hexenyl benzoate **129** Hexyl acetate **130** α-Humulene **131** Humulene oxide **132** α-Ionone **133** Isocaryophyllene **134** Junenol **135** Limonene **136** Linalool **137** 1-Methylcyclohexene **138** (*E*)-6-Methyl-3,5-heptadien-2-one **139** 6-Methyl-5-hepten-2-one **140** 3-Methyl-2-pentanone **141** Methyl-4-methoxybenzoate **142** 4-Methoxybenzaldehyde **143** T-muurolol **144** Myrcene **145** (*E*)-Nerolidol **146** Nonanal **147** Nonanoic acid **148** Nonanol **149** (*E*)-β-Ocimene **150** Octanal **151** *n*-Octane **152** Octanol **153** α-Selinene **154** β-Selinene **155** β-Sesquiphellandrene **156** Spathulenol **157** Styrene **158** α-Terpineol **159** Toluene **160**	

***Mesua* sp.**	**Plant part**	**Compounds**	**References**
		α-Ylangene **161**	
Mesua myrtifolia (Baill.) Kosterm.	Bark	Betulinic acid **23** Jacareubin **162** Myrtifolic acid **163** Oleanolic acid **164** Simiarenone **165** Simiarenol **166** β-Sitosterol **62** Taraxerol **167**	[75]
Mesua racemose (Planch. ex Triana et Planch) Kosterm.	Leaves	Furanoracemosone **168** Isoracemosol **169** Mammea A/AA or mammeisin **71** Mammea A/AC **170** Mammea A/AC cyclo D **171** Mammea A/AC cyclo F **13** Mammea A/BB or isomammeisin **55** Mammea A/BC **172** Mammea A/AD cyclo D or mesuagin **74** Racemosol **12** Racemosone **173**	[76,47]
Mesua thwaitesii Planch. & Triana	Bark	Mammea A/AA cyclo F **52** Mammea A/AB cyclo F **53** Mesuaxanthone B **61** β-Sitosterol **62**	[77]
	Seeds	Mammea A/AA or mammeisin **71** Mammea A/AA cyclo D **51** Mammea A/AA cyclo F **52** Mammea A/AB **72** Mammea A/AB cyclo D or mammeigin **73** Mammea A/AD cyclo D or mesuagin **74**	[77]

***Mesua* sp.**	**Plant part**	**Compounds**	**References**
		Mammea A/AD cyclo F **54** Mesuol **76**	
	Timber	1,3-Dimethoxy-5-hydroxyxanthone **174** 1,5-Dihydroxyxanthone **57** Euxanthone **18**	[77]



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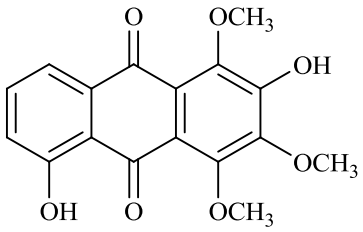

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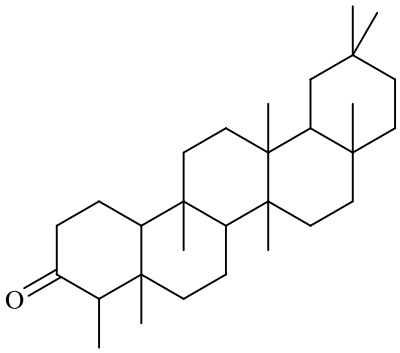

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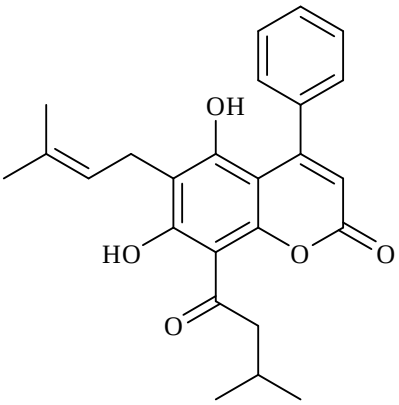

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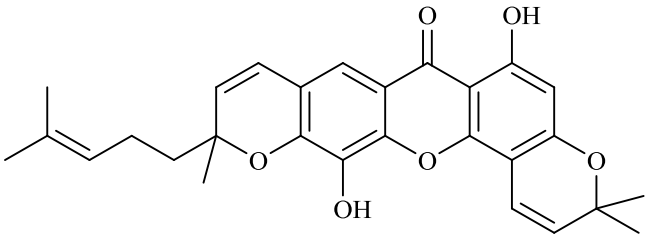

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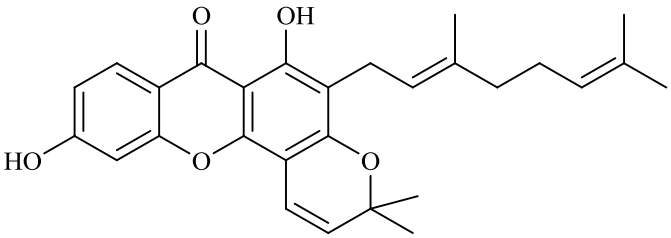

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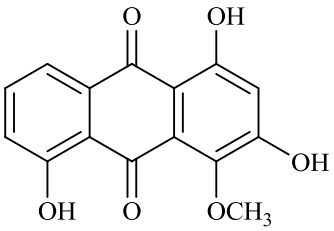

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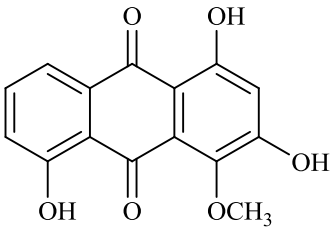

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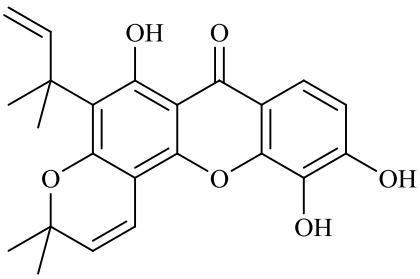

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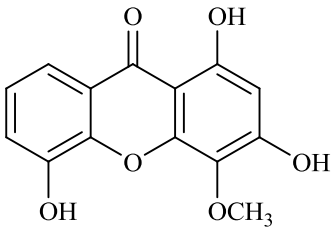

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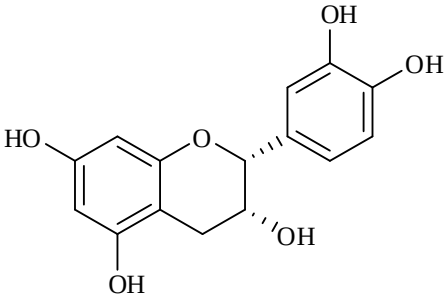

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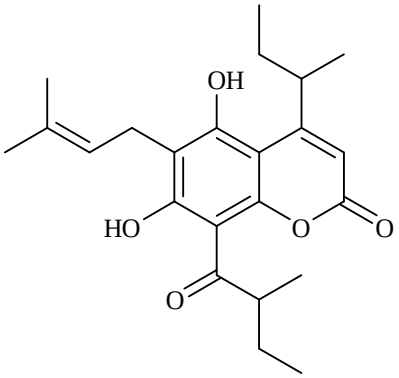

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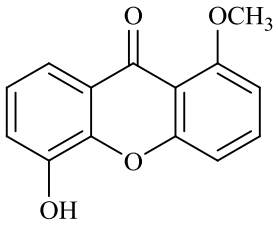

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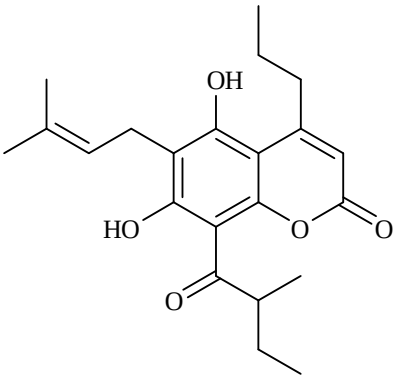

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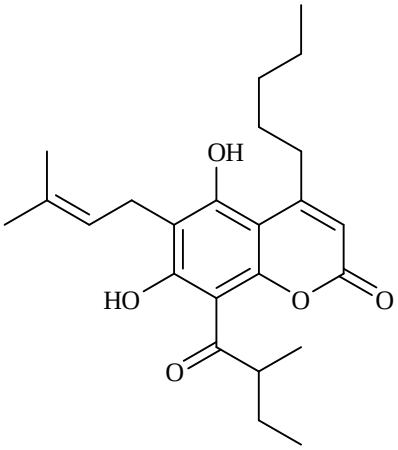

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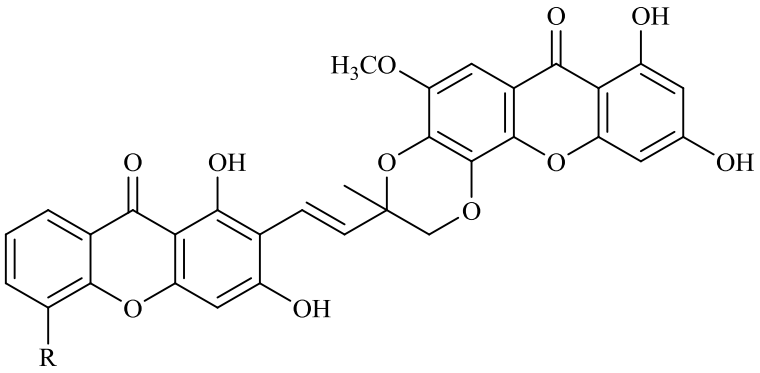
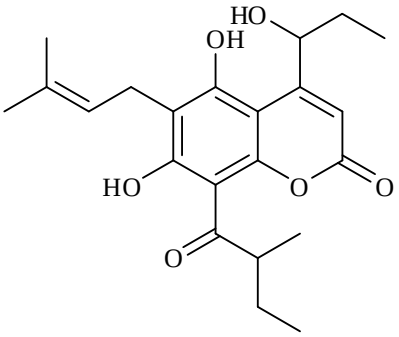


40 R = OH

41 R = OCH_3



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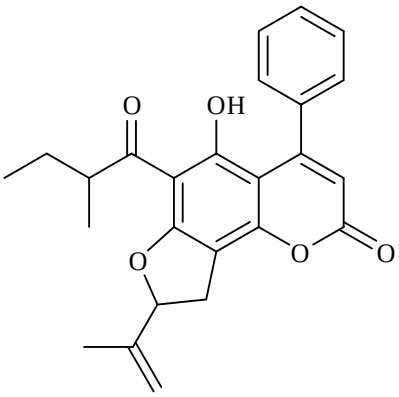

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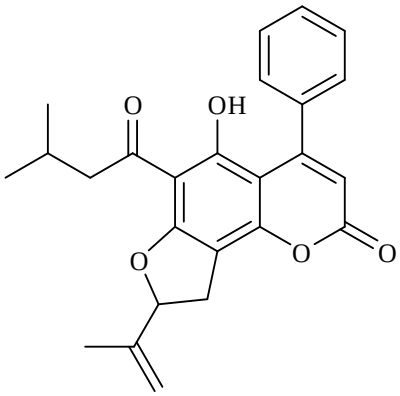

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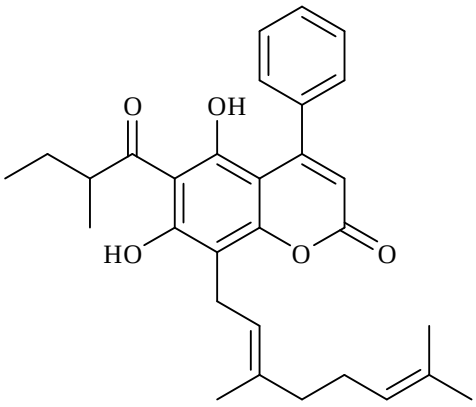

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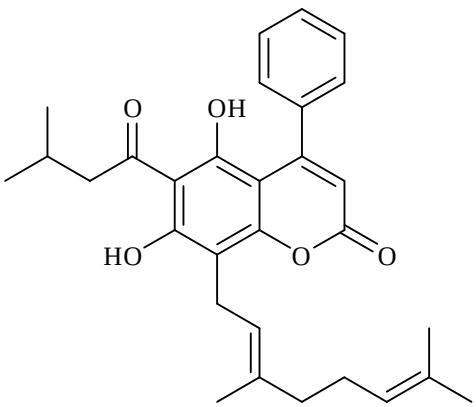

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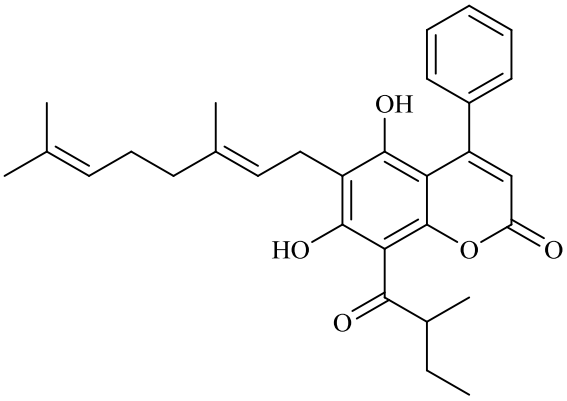

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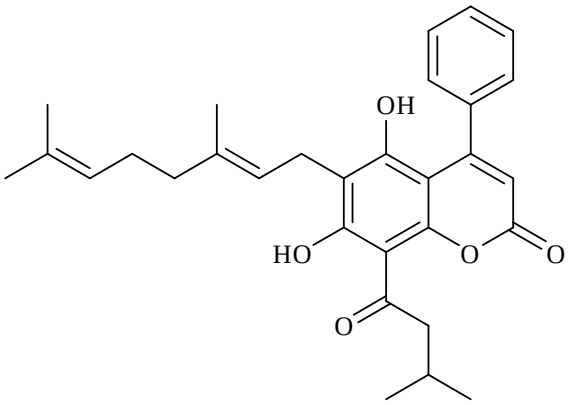

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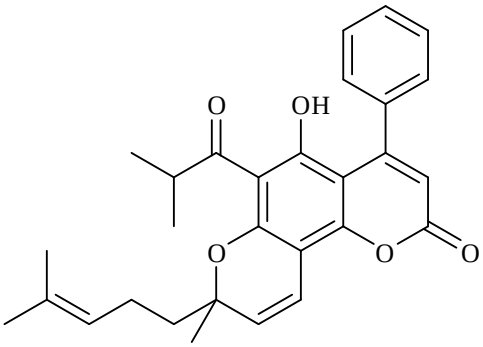

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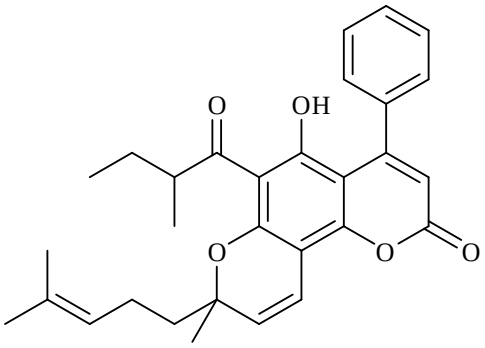

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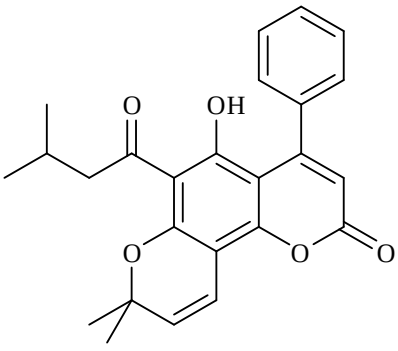

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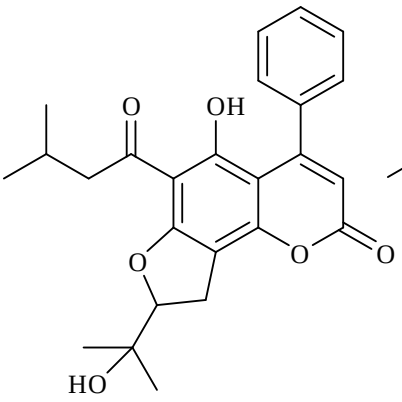

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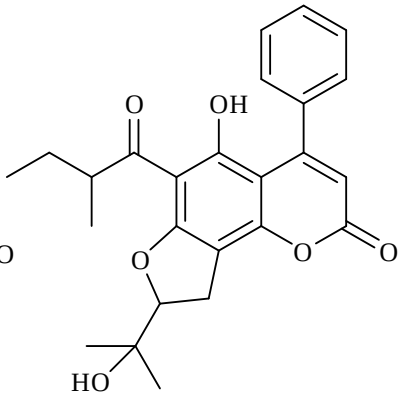

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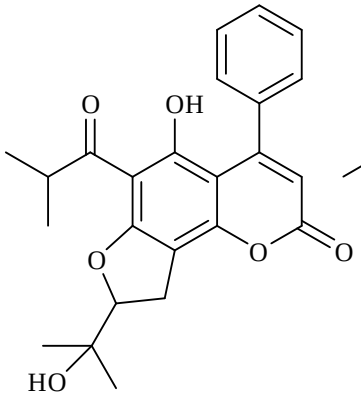

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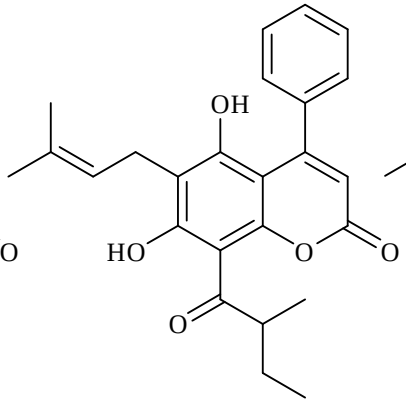

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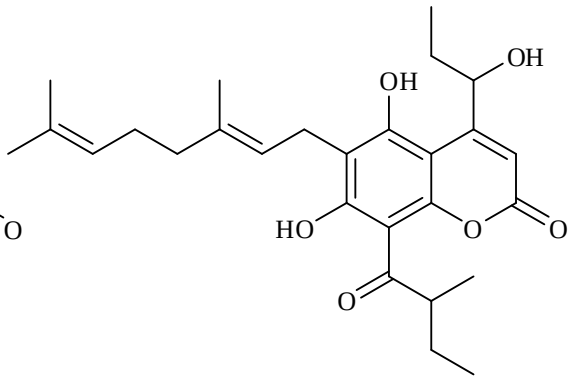

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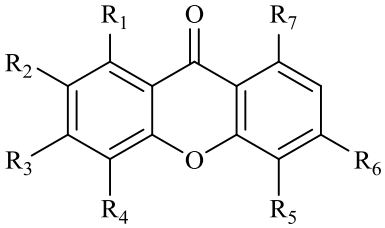
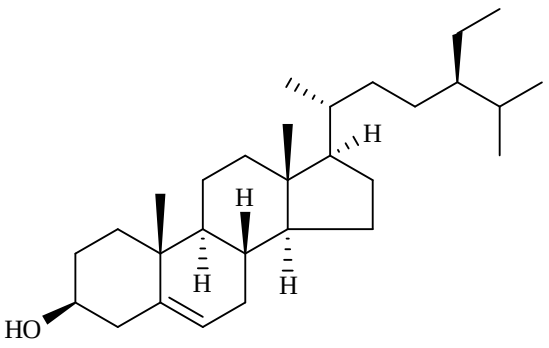


	R_1	R_2	R_3	R_4	R_5	R_6	R_7
57	H	H	H	OH	H	H	OH
58	H	OCH_3	H	H	H	H	OH
59	OCH_3	H	OCH_3	H	OH	OH	H
60	H	H	H	OH	H	OCH_3	OH
61	H	H	OH	OH	H	H	OH



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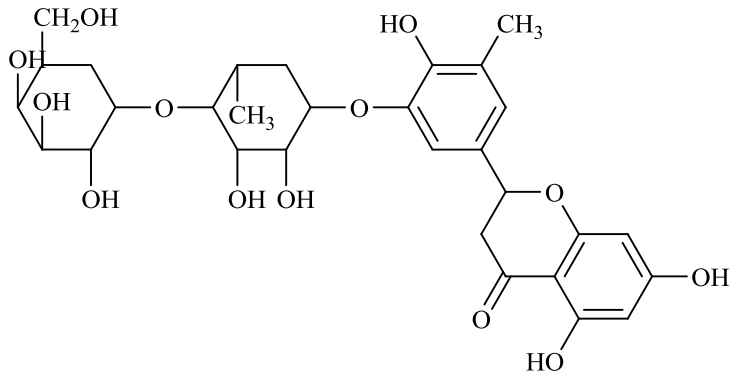

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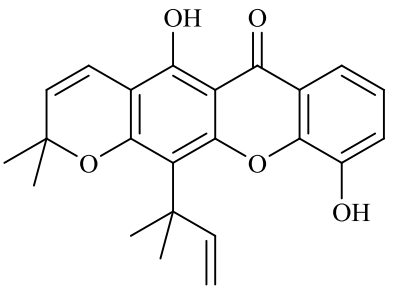

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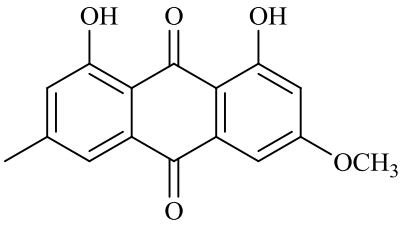

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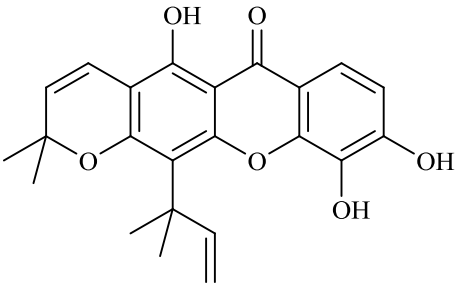

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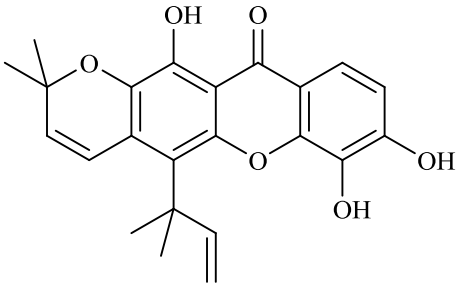

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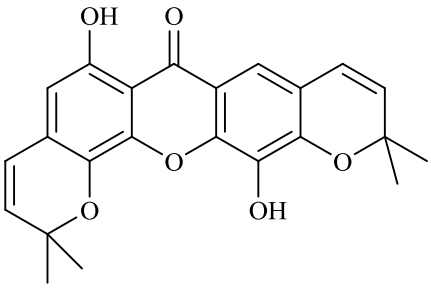

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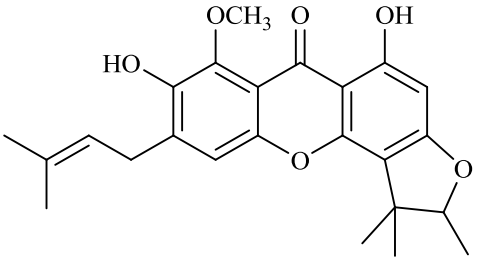

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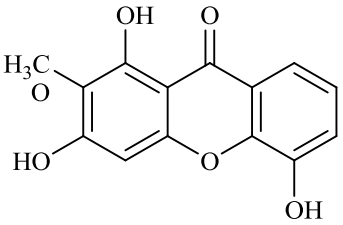

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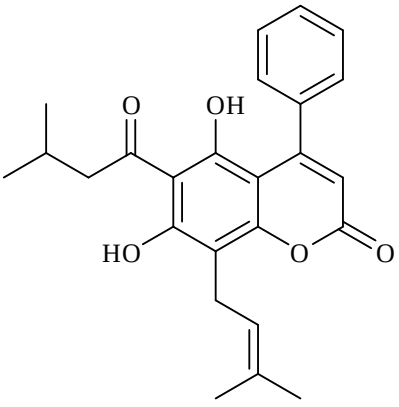

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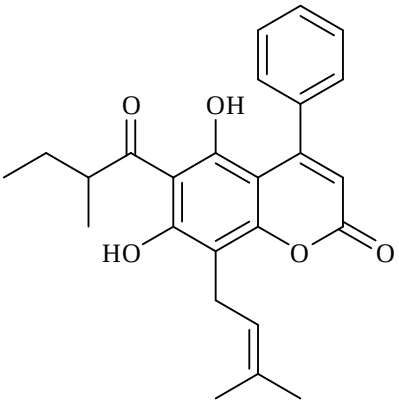

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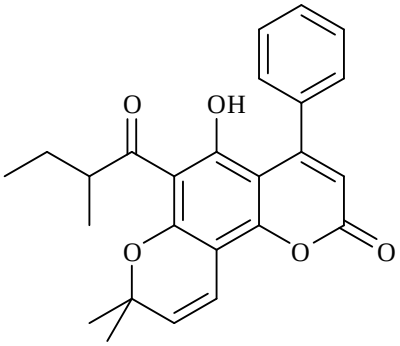

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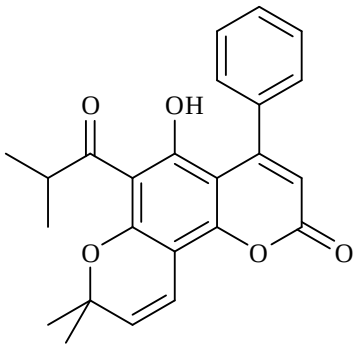

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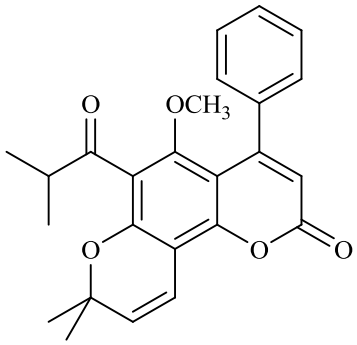

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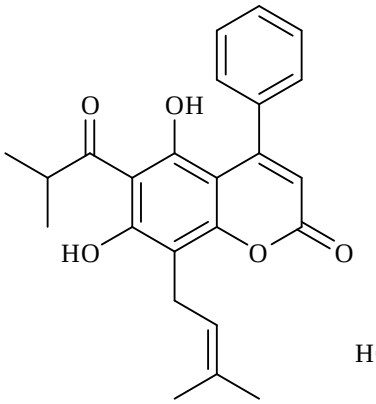

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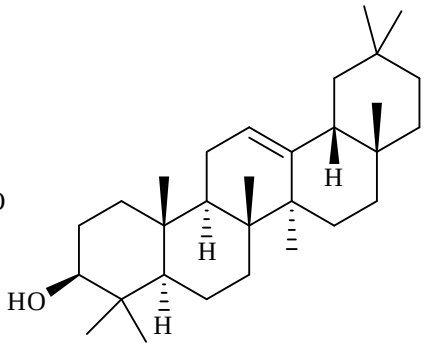

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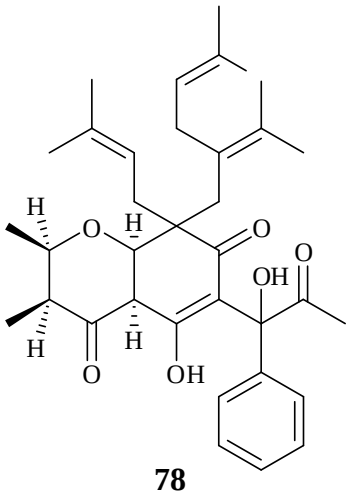

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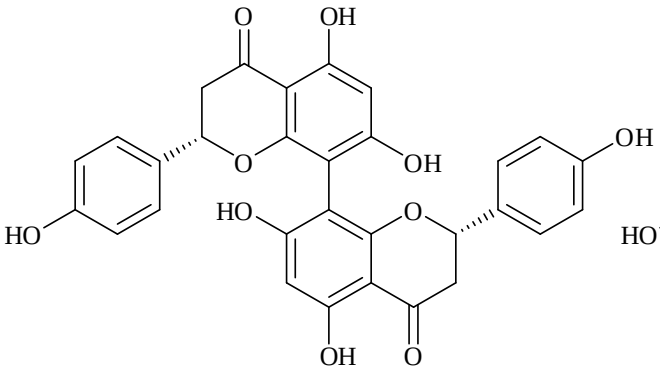

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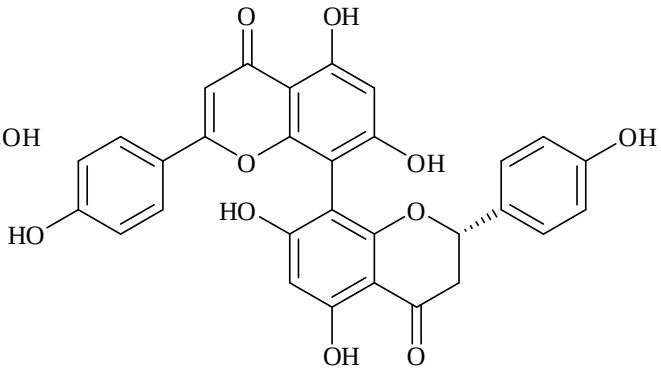

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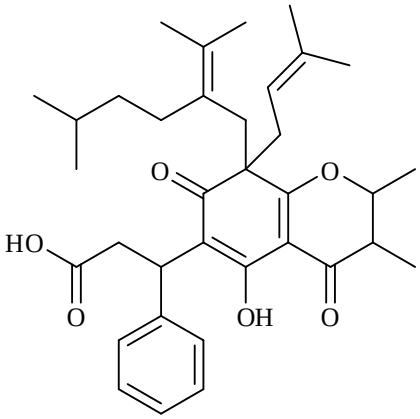

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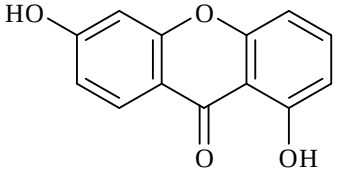

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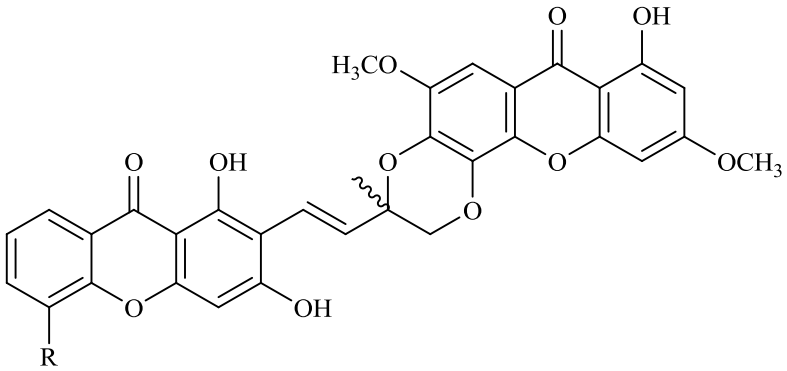
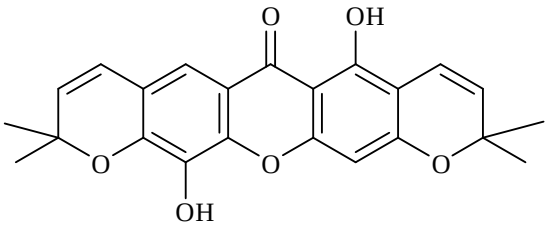


83 R = OH

84 R = OCH_3



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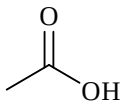

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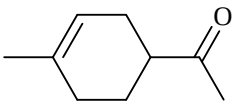

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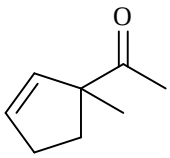

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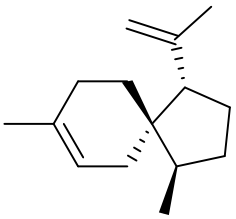

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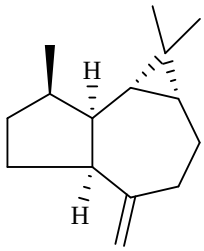

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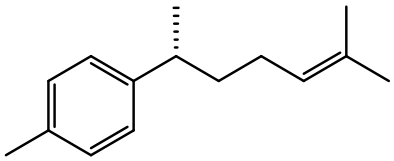

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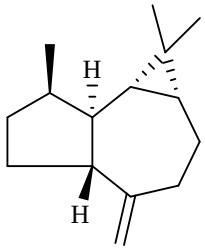

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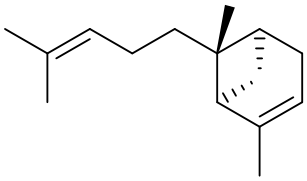

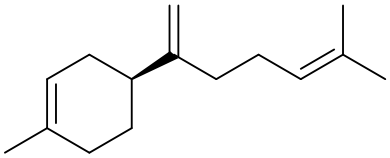

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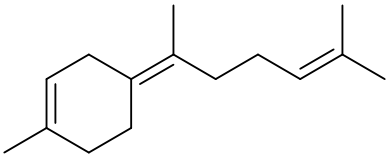

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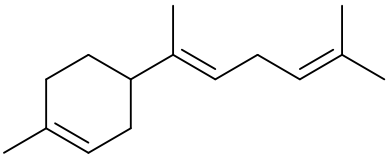

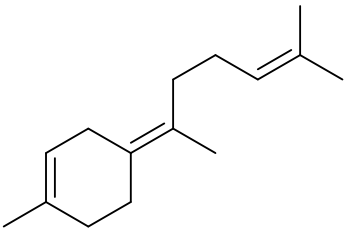

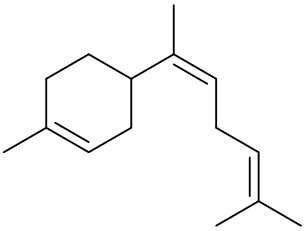

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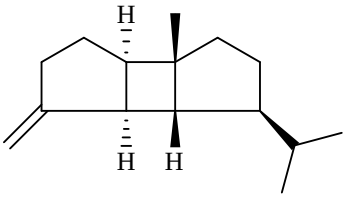

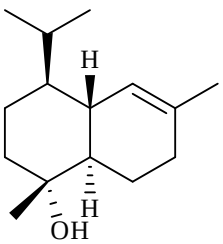

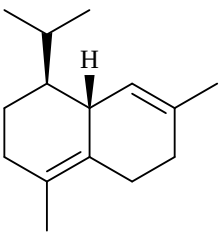

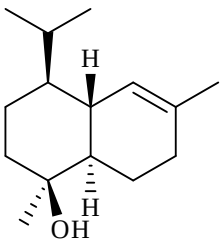

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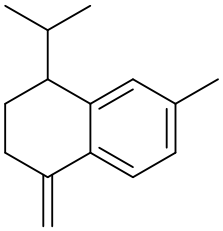

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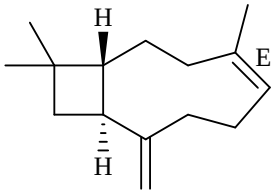

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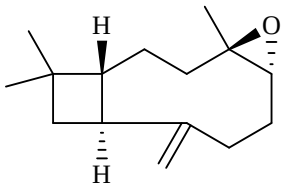

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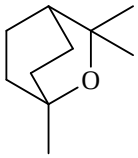

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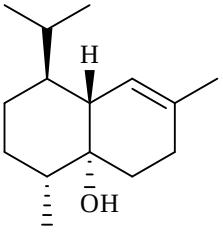

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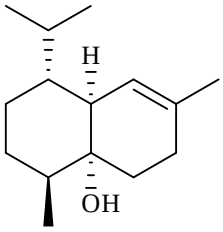

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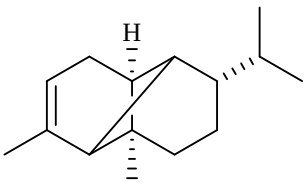

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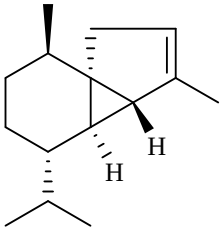

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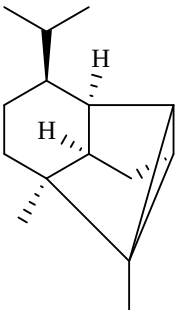

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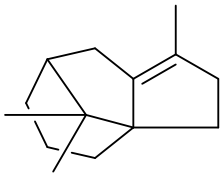

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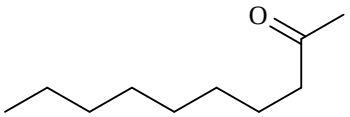

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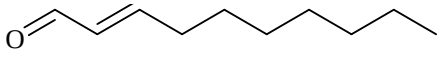

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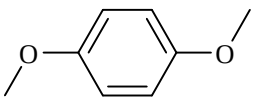

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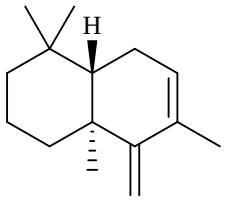

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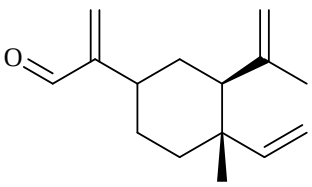

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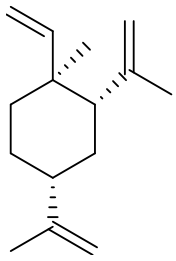

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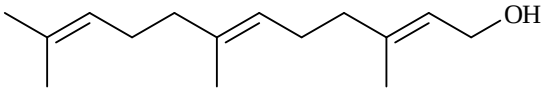

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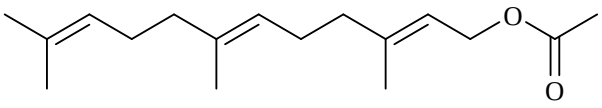

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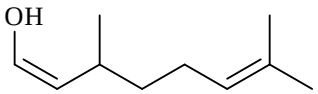

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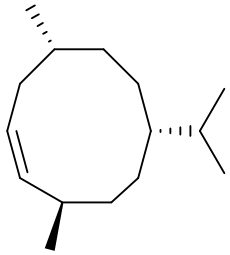

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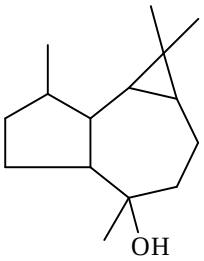

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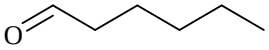

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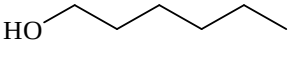

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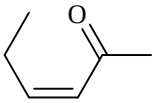

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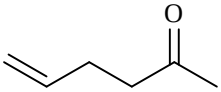

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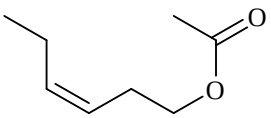

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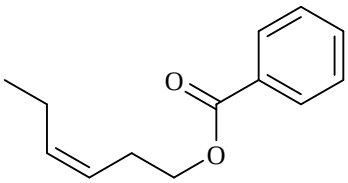

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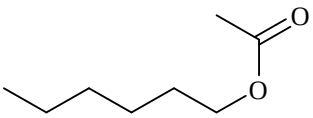

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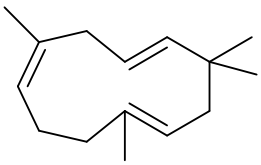

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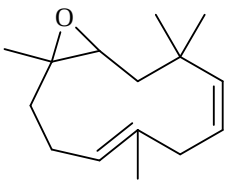

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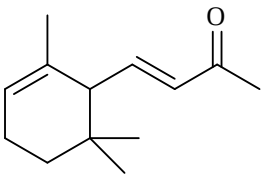

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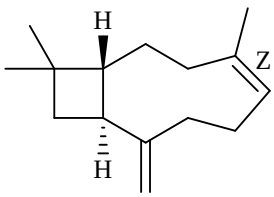

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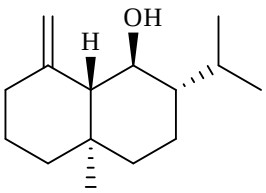

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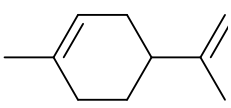

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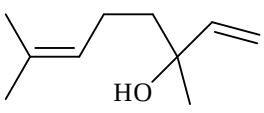

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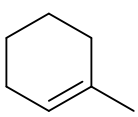

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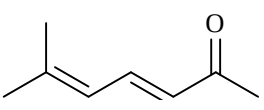

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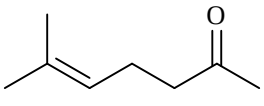

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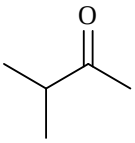

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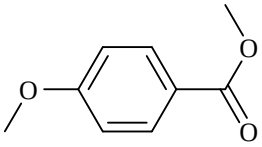

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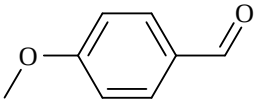

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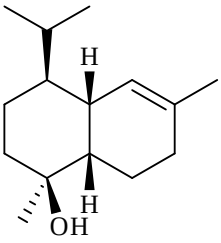

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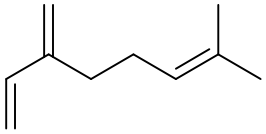

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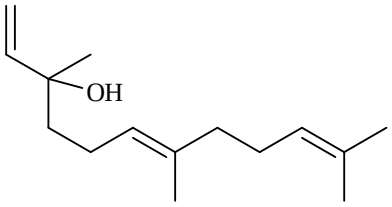

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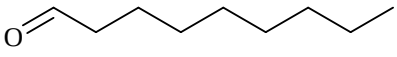

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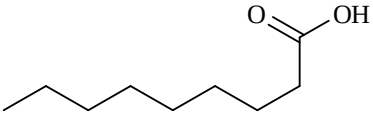

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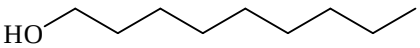

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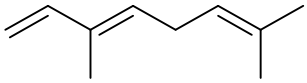

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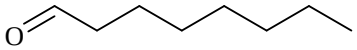

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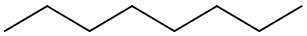

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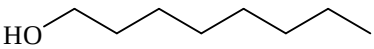

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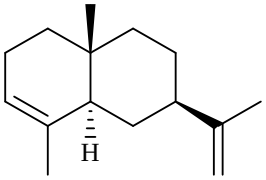

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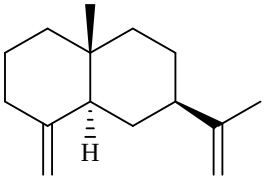

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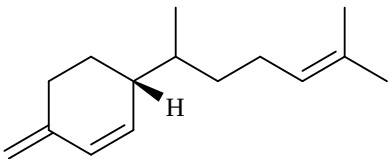

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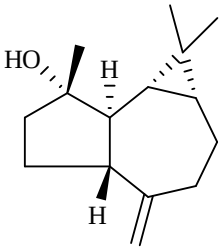

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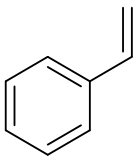

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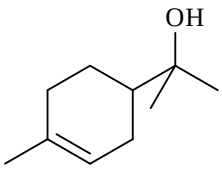

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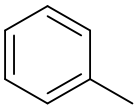

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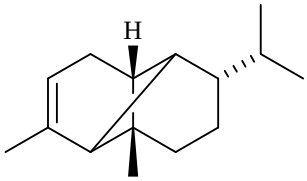

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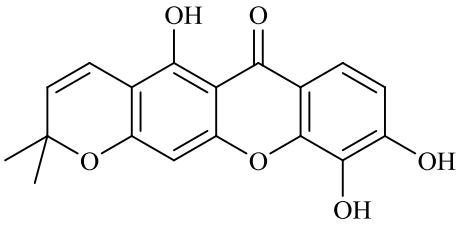

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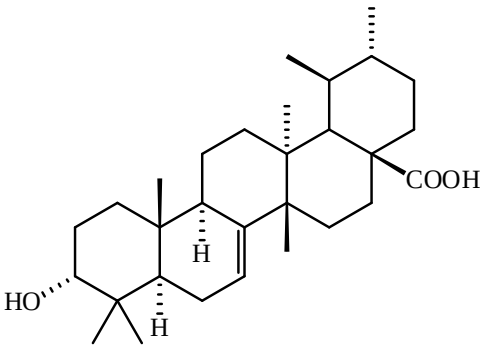

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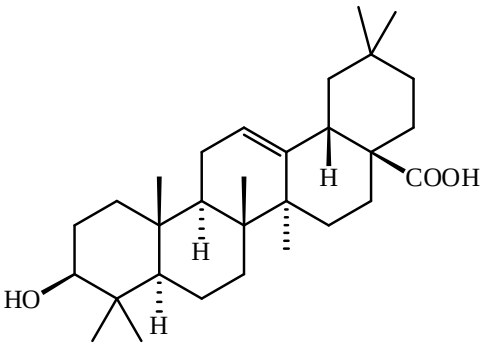

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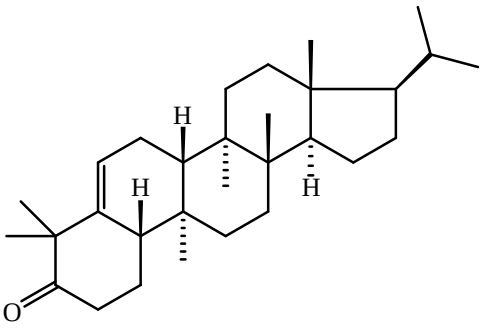

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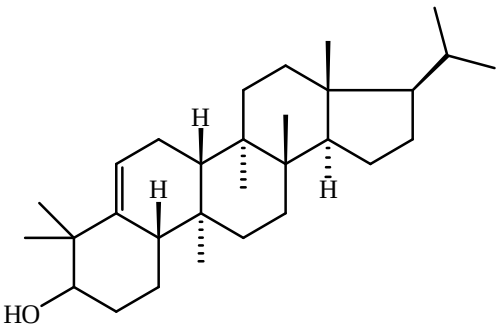

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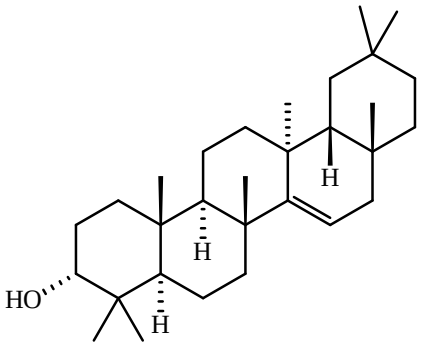

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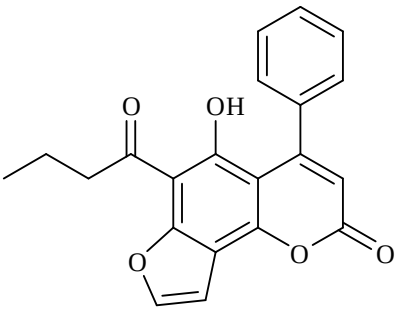

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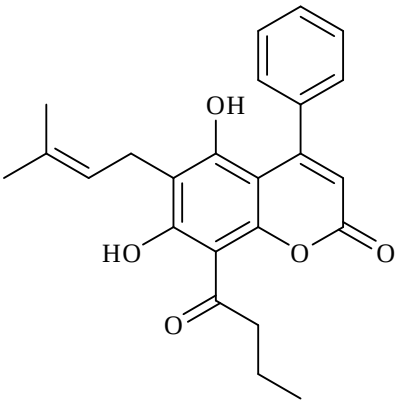

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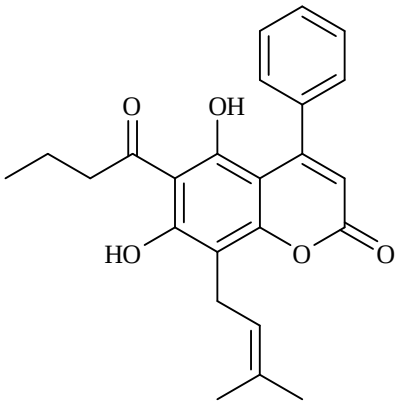

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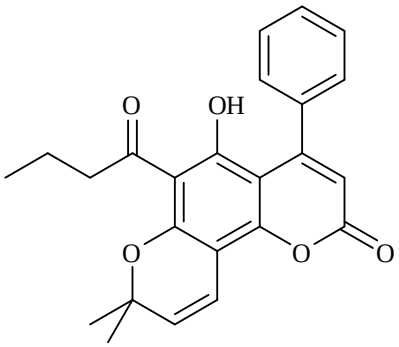

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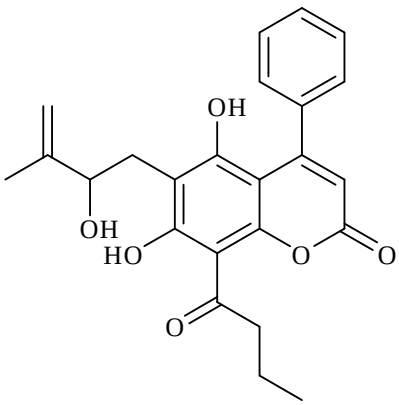

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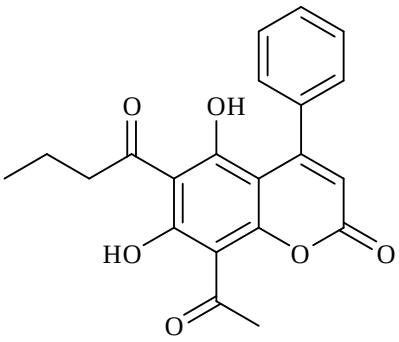

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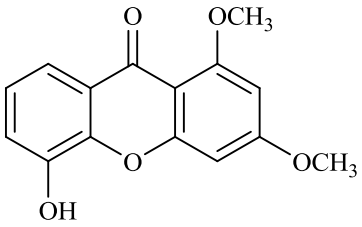


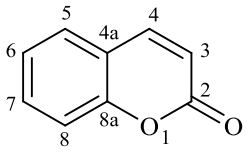
174

2.1 General Chemical Aspects of Coumarins

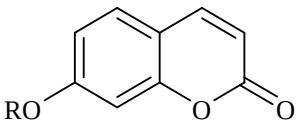
Mesua plants are known producers of various compounds, notably coumarins and xanthones. In this study, both *Mesua* species studies yielded prenylated and geranylated 4-phenylcoumarins. Therefore, the following sub-chapters will discuss briefly the biosynthesis and characteristics of the coumarins, particularly the 4-phenylcoumarins (neoflavonoids).

2.1.1 Coumarins

Coumarins possess the nucleus 2*H*-1-benzopyran-2-one which may be considered, on first approximation, as lactone of 2'-hydroxy-*Z*-cinnamic acid[78]. Since the discovery by Vogel in 1820 of the simplest member of the class, coumarin itself **175,** in *Dipteryx odorata* Willd. (Fabaceae) (the tonka bean), this compound and a wide range of its structural derivatives have been shown to occur in hundreds of plant species[79]. More than one thousand coumarins have been described, and the simplest among them are widely distributed throughout the vegetable kingdom[78].



Coumarin **175**



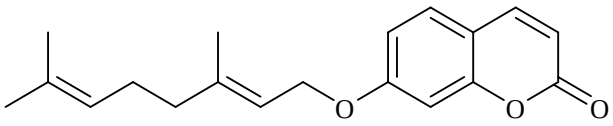
176 R = H

177 R = Me

Except for a few rare cases, all coumarins are substituted by a hydroxyl group in position 7. Umbelliferone **176** which is 7-hydroxycoumarin itself, is the precursor of the 6,7-di- and 6,7,8-trihydroxylated coumarins. The hydroxyl group of these simple coumarins are either methylated, or sometimes one of them is engaged in glycosidic linkage. Another structural feature which is common to many coumarins is prenylation

and geranylation; *O*-prenylation or *O*-geranylation, or more frequently, prenylation or geranylation on the ring in the 6- or 8-position of umbelliferone **176** or herniarin **177**, which gives rise to aurapten **178**, suberosin **179** and osthol **180**[78].

There may even be a five-carbon residue on the 3-position, although to a much lesser extent. The high reactivity of the isoprene chain (C_5, C_{10}, or more rarely C_{15}) explains the large number of derived structures (epoxidized, mono- and dihydroxylated, and cyclised). Prenylation and geranylation are also the origin of the polycyclic coumarins, the furano- and pyranocoumarins, and the linear (e.g. imperatorin **181** and xanthyletin **182**), and angular (e.g. angelicin **183** and visnadin **184**) coumarins[78].



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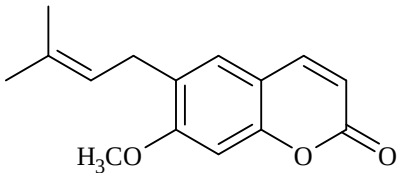

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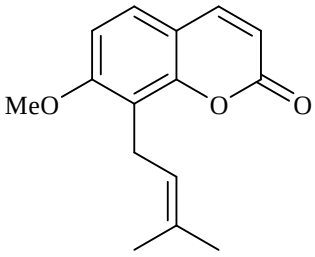

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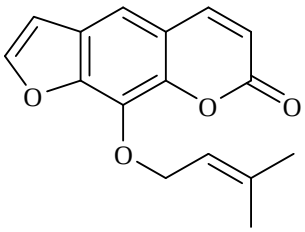

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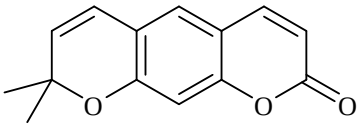

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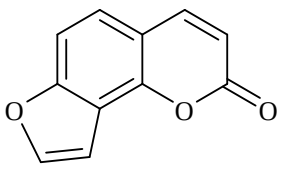

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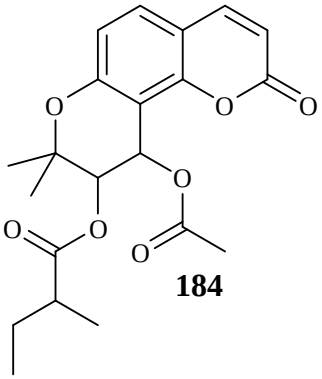
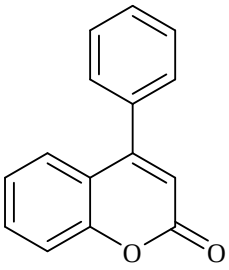


184

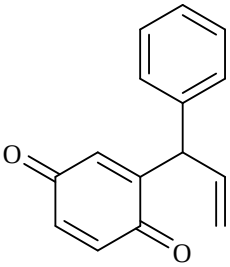
2.1.2 4-Phenylcoumarins – Neoflavonoids

The term neoflavonoid refers to a group of C_{15} naturally-occurring compounds having a C_6-C_3-C_6 skeleton, structurally related to flavonoids and isoflavonoids, and constructed on a 1,1-diphenylpropane skeleton[78]. This term was suggested by Swain to describe the group of natural products with a 4-phenylchroman skeleton[80].

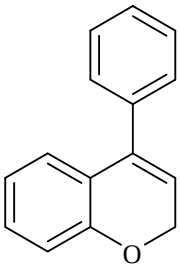
Like the 2- and 3-phenylchromanes, they arise from the condensation of three acetate molecules with one cinnamate molecule, but their mechanism of formation is very different. It is possible that they arise from the reaction of a phenol such as phloroglucinol or resorcinol with the α-carbon of the side chain of a phenylpropane unit. The vast majority of known neoflavonoids have been encountered in the Fabaceae and Clusiaceae. Some are also present in the Rubiaceae[78]. Scheme 2.1 shows the naturally occurring neoflavonoids that have been arranged conveniently (although arbitrarily) in groups according to their structural type, as proposed by Donnelly in 1975[80].



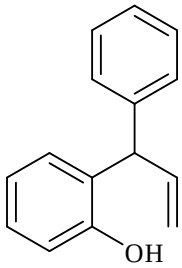
4-Arylcoumarin



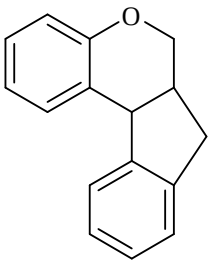
Dalbergione



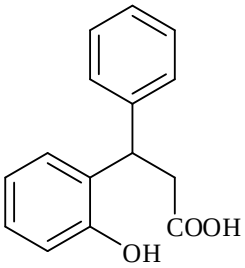
Neoflavene



Dalbergiquinol



4-Arylchroman

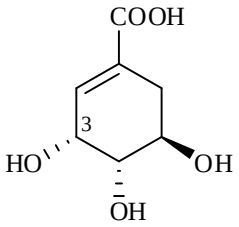


Coumarinic Acid

Scheme 2.1: Examples of Neoflavonoid Skeletons

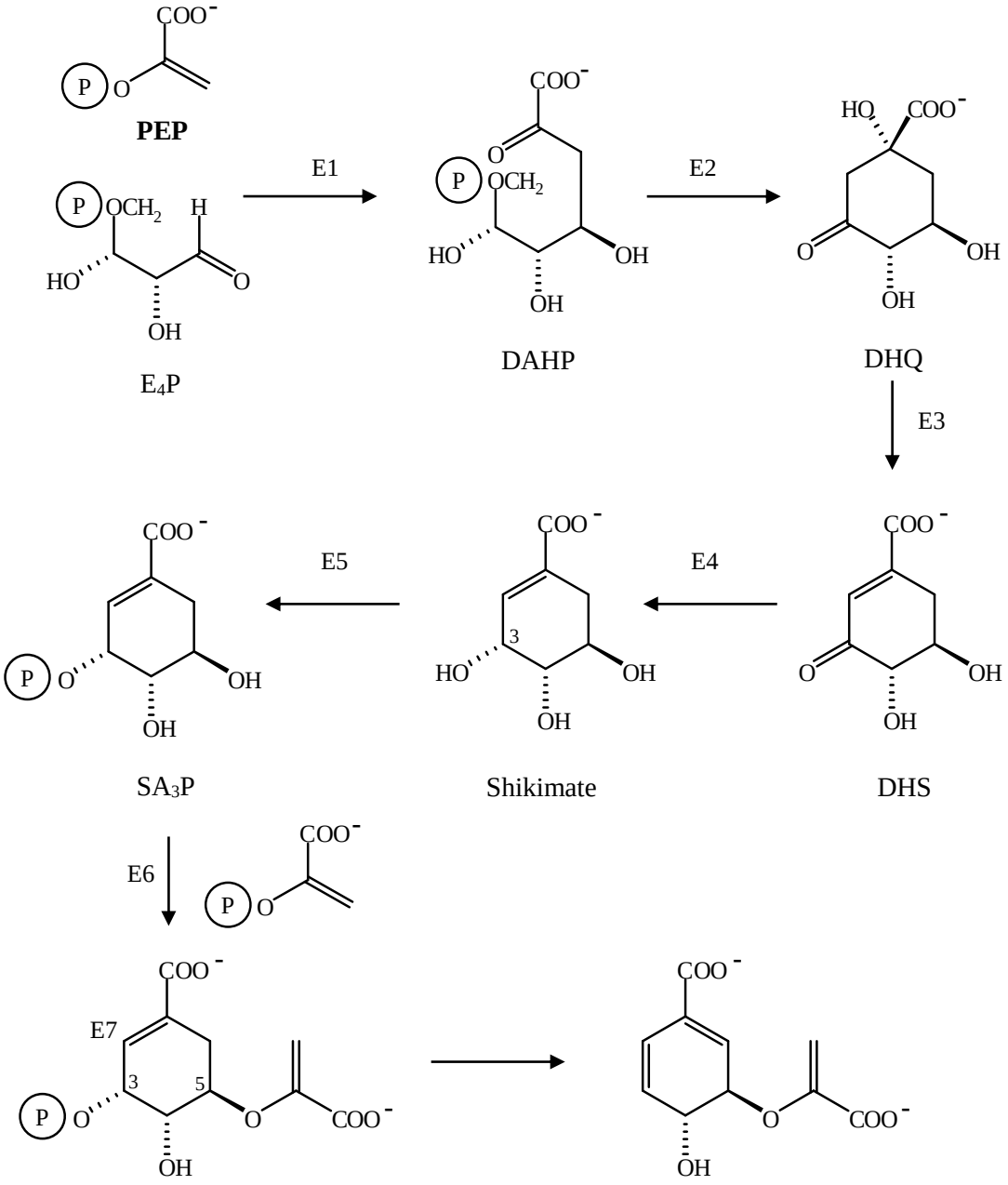
2.2 Biosynthesis of Coumarins and 4-Phenylcoumarins

Coumarins in higher plants have been known to derive from the shikimate pathway. The shikimate pathway is a major route leading to the formation of aromatic compounds in living systems. The structure of shikimic acid **185** is shown below, after which the pathway was named.



Shikimic acid **185**

The building blocks of shikimic acid are phosphoenolpyruvate (PEP) and D-erythrose-4-phosphate (E_4P). These combine in a reaction catalyzed by the enzyme DAHP synthase to give the 7-carbon sugar; 3-deoxy-D-arabino-heptulosonic acid-7-phosphate, which is subsequently cyclised to the first carbocyclic compound, 3-dehydroquinate (DHQ). Hydrolysis of the latter gives 3-dehydroshikimate (DHS), followed by reduction of the carbonyl functional group to give shikimate, which is subsequently phosphorylated in the position-3 to form shikimate 3-phosphate (SA_3P). Attachment of a 3-carbon side chain, provided by another molecule of phosphoenolpyruvate leads to the formation of 5-enolpyruvylshikimate-3-phosphate (EPSP), followed by a 1,4-elimination of the elements of phosphoric acid to form of chorismate[81]. These processes are illustrated in Scheme 2.2.



Scheme 2.2: The Shikimate to Chorismate Biosynthetic Pathway

E1: 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase(DAHP syntase/synthetase)

E2: 3-Dehydroquinate synthetase

E3: 3-Dehydroquinate hydro-lyase (DHQase)

E4: Shikimate dehydrogenase (SHorase)

E5: Shikimate kinase

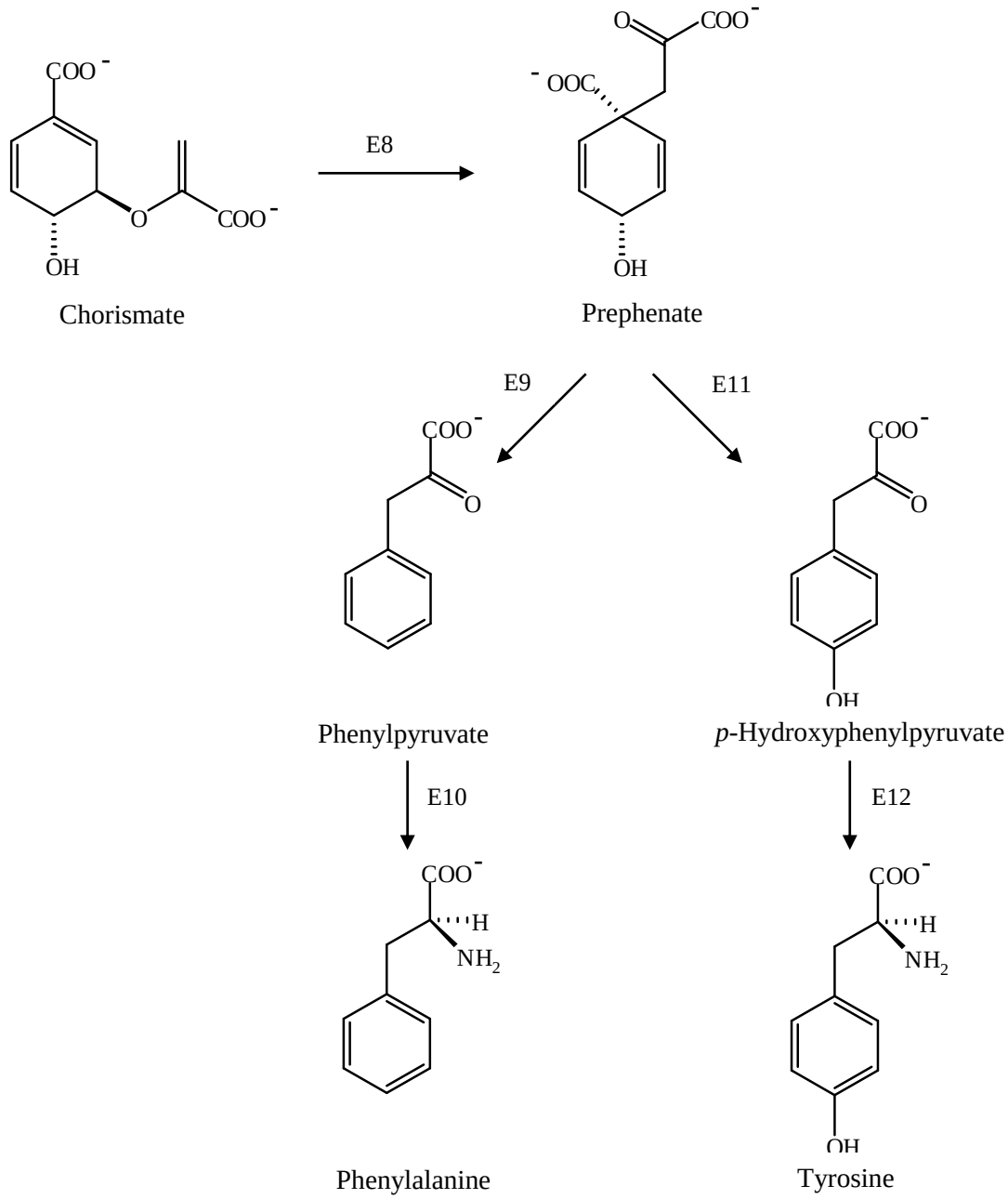
E6: 5-Enolpyruvylshikimate-3-phosphate synthase (EPS synthase)

E7: Chorismate synthase

Chorismate, the product of the shikimate pathway is the key compound for the formation of coumarin through Claisen rearrangement, which leads to the formation of phenylalanine and tyrosine (Scheme 2.3). The committed step in the formation of coumarin is the *ortho*-hydroxylation of a *trans*-cinnamic acid, which is derived from phenylalanine by the action of phenylalanine ammonia-lyase (Scheme 2.4)[81].

The addition of a prenyl group at the C-6 or C-8 position of coumarin also often occurs[79]. Prenylation of the benzene ring by dimethylallyl pyrophosphate (DMAPP) in the 6- or 8-position of a 7-hydroxycoumarin is the origin of the extra ring which characterizes these molecules. Prenylation in the 6-position yields the so-called 'linear' furano- or pyranocoumarins; in the 8-position it affords the 'angular' homologs[78].

The cyclization of 6- or 8-isoprenylcoumarin is probably due to nucleophilic attack by the hydroxyl group in the 7-position on an epoxide formed by oxidation of the double bond of the isopentenyl residue. The product of this reaction depends on the orientation of the nucleophilic attack. It is either a 2-hydroxyisopropy-ldihydrofuranocoumarin, or, in the case of an attack on the tertiary carbon, a hydroxydimethyl-dihydropyranocoumarin[78].



Scheme 2.3: Pathways to Phenylalanine and Tyrosine

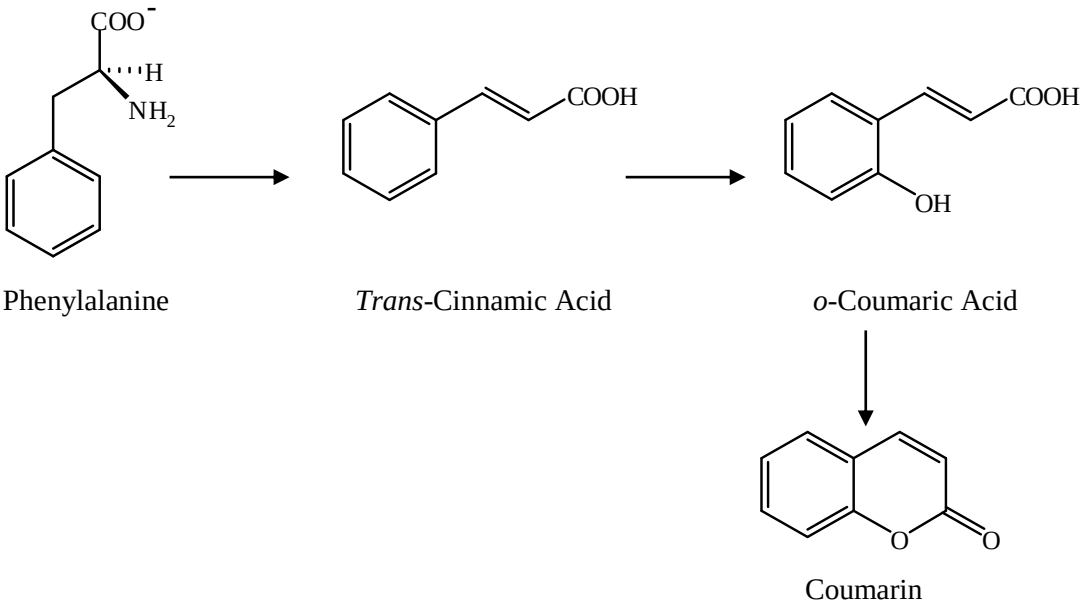
E8 : Chorismate mutase

E9 : Prephenate dehydratase

E10 : Phenylalanine aminotransferase

E11 : Prephenate dehydrogenase

E12 : Tyrosine aminotransferase

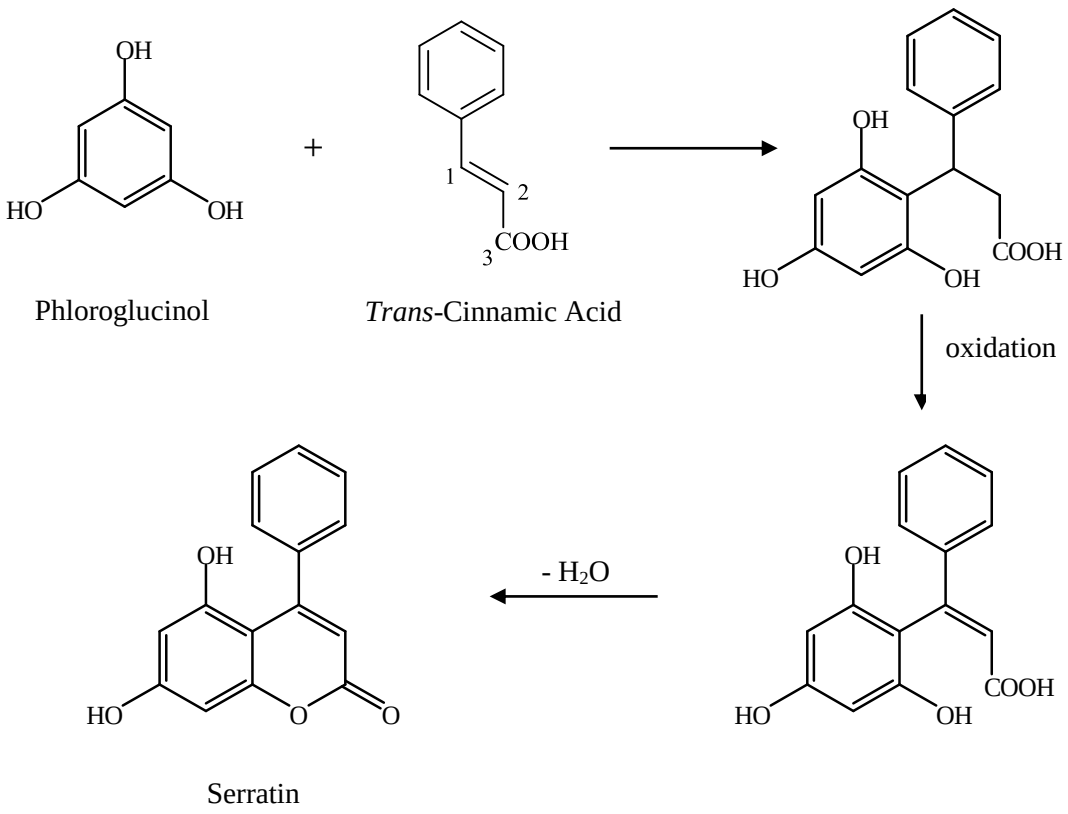


Phenylalanine *Trans*-Cinnamic Acid *o*-Coumaric Acid

Coumarin

Scheme 2.4: Biosynthetic Pathway from Phenylalanine to Coumarin

Coumarins with a phenyl substituent at carbon 3 or 4 are formed through an entirely different route from those discussed so far. In the case of 4-phenylcoumarins, Kunesh and Polonsky in 1967 showed that the benzene ring of the benzopyran nucleus derives from acetate, not shikimate, and that the remaining nine carbons are shikimate-derived. The coumarin nucleus of calophyllolide, for example, a 4-phenylcoumarin from *Calophyllum inophyllum* L., originates through a condensation involving C-3 of a phenylpropanoid and an acetate-derived phenol. The molecule formed then undergoes reduction followed by cyclization through the loss of water to give 4-phenylcoumarin. The proposed biosynthetic pathway to 4-phenylcoumarins is shown in Scheme 2.5[79].



Phloroglucinol *Trans*-Cinnamic Acid

Serratin

Scheme 2.5: Biosynthetic Pathway to 4-Phenylcoumarin

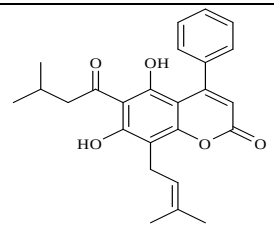
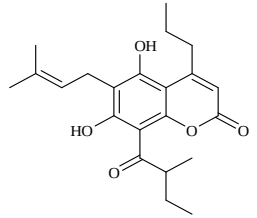
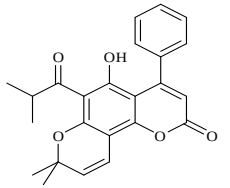
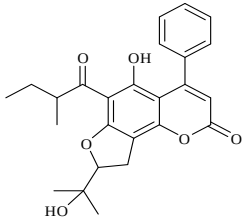
2.3 The Naming System of Coumarins

To avoid the proliferation of very similar trivial names, a naming system for the so-called mammea coumarins were introduced by Crombie *et al*., when the author was working with *Mammea americana*[82]. Within each group, as well as a 5,7-dioxygenated pattern, the aromatic ring carries an acyl and a prenyl group. The name mammea is followed by a first letter designating the 4-substituent, and a stroke separates this from a second letter designating whether a 6-acyl or 8-acyl group is present. A third letter designates the type of acyl substituent. Where the prenyl substituent has been modified by cyclisation, the third letter is followed by the prefix cyclo and a fourth letter indicates the type of heterocyclisation. Table 2.2 below shows a summary of the coding system. Some examples of coumarin derivatives and their naming system are described in Table 2.3.

Table 2.2: Summary of the Naming System for the Mammea Type Coumarins

1st Letter	**2nd Letter**	**3rd Letter**	**4th Letter (if cyclisation occurs)**
A = phenyl B = propyl C = pentyl D = 1-methylpropyl E = 1-acetoxypropyl	A = 6-acyl B = 8-acyl	A = 3-methylbutyryl B = 2-methylbutyryl C = butyryl D = 2-methylpropionyl	cyclo D = 2,2-dimethylchromene cyclo E = 3-hydroxy-2,2-dimethyldihydropyran cyclo F = 2-(1-hydroxy-1-methylethyl)dihydrofuran

Table 2.3: Examples of Coumarin Derivatives and Their Naming System

Compound	**1st Letter**	**2nd Letter**	**3rd Letter**	**4th Letter (if cyclisation occurs)**	**Structure**
Mammea A/AA **71**	A = phenyl	A = 6-acyl	A =3-methylbutyryl	-	
Mammea B/BB **38**	B = propyl	B = 8-acyl	B =2-methylbutyryl	-	
Mammea A/AD cyclo D **74**	A = phenyl	A = 6-acyl	D =2-methylpropionyl	cyclo D = 2,2-dimethylchromene	
Mammea A/AB cyclo F **53**	A = phenyl	A = 6-acyl	B = 2-methylbutyryl	cyclo F = 2-(1-hydroxy-1-methylethyl)dihydrofuran	

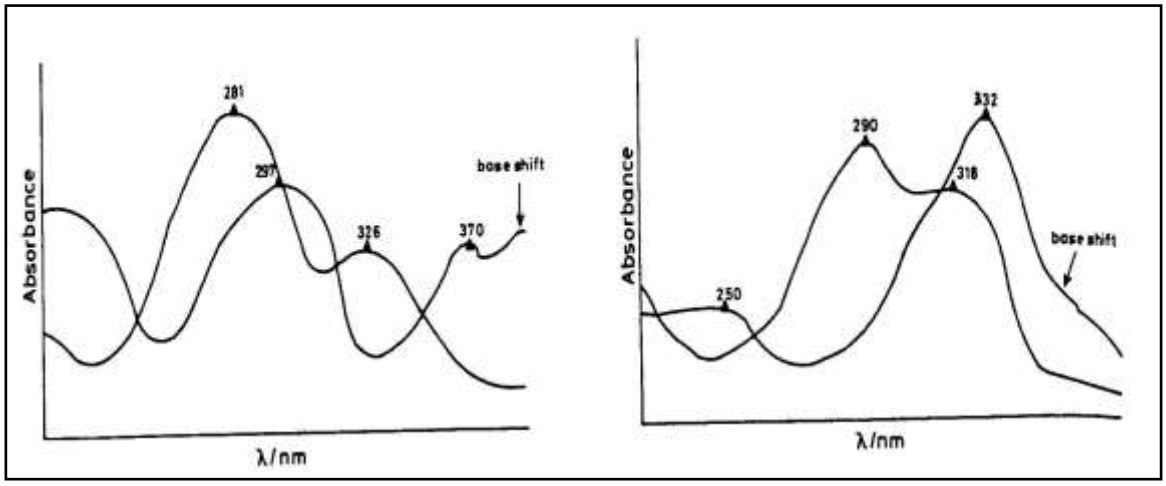
2.4 Structural Elucidation: General Methods and Theory

Various methods are being used by natural product chemists to identify and structurally elucidate the isolated chemical constituents. Amongst the methods being applied include the ultraviolet (UV), infrared (IR), mass spectrometry (MS), proton nuclear magnetic resonance (^{1}H NMR) and carbon nuclear magnetic resonance (^{13}C NMR).

2.4.1 Ultraviolet Spectra of Coumarins

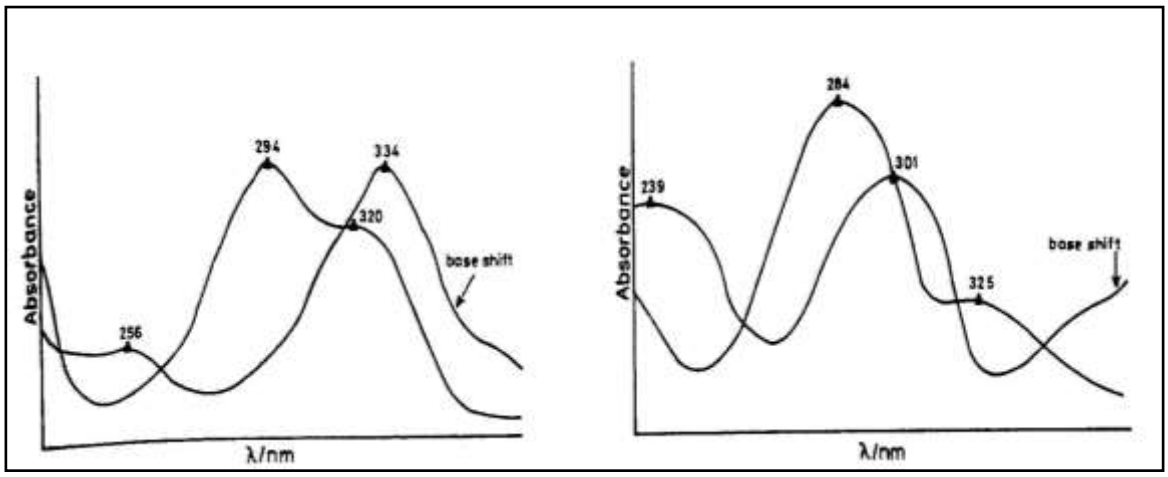
Coumarins have characteristic UV spectra which are heavily influenced by the nature and the position of substituents, and by alkalinisation (KOH, $NaOCH_3$) if a phenolic group is present[78]. A coumarin has UV absorption bands at λ_{max} 212, 274, 282 and 312 nm, while a 7-hydroxycoumarin (umbelliferone **176**) has characteristic UV absorptions at λ_{max} 210, 240 and 325nm[83].

The orientation of the acyl group in substituted coumarins can be assigned based on UV absorption data. The UV spectra of the 6-acylcoumarins are consistent, irrespective of the acyl group and the C-4 substituent, and the same applies to 8-acylcoumarins. Typical spectra in ethanol and with base-shift, for a 6-acyl and 8-acyl isomer are shown in Figures 2.1 and 2.2, respectively. The base-shifts are characteristically different, and in addition, the presence of a λ_{max} at 281 ± 1nm in the 6-acyl isomers, and of a λ_{max} at 290 ± 1nm in the 8-acyl isomers, is the most useful aid to the rapid differentiation of isomers without resorting to a base-shift[82].



(i) (ii)

Figure 2.1: Typical UV Spectrum of (i) 6-Acyl-5,7-dihydroxycoumarin and (ii) 8-Acyl-5,7-dihydroxycoumarin in Ethanol

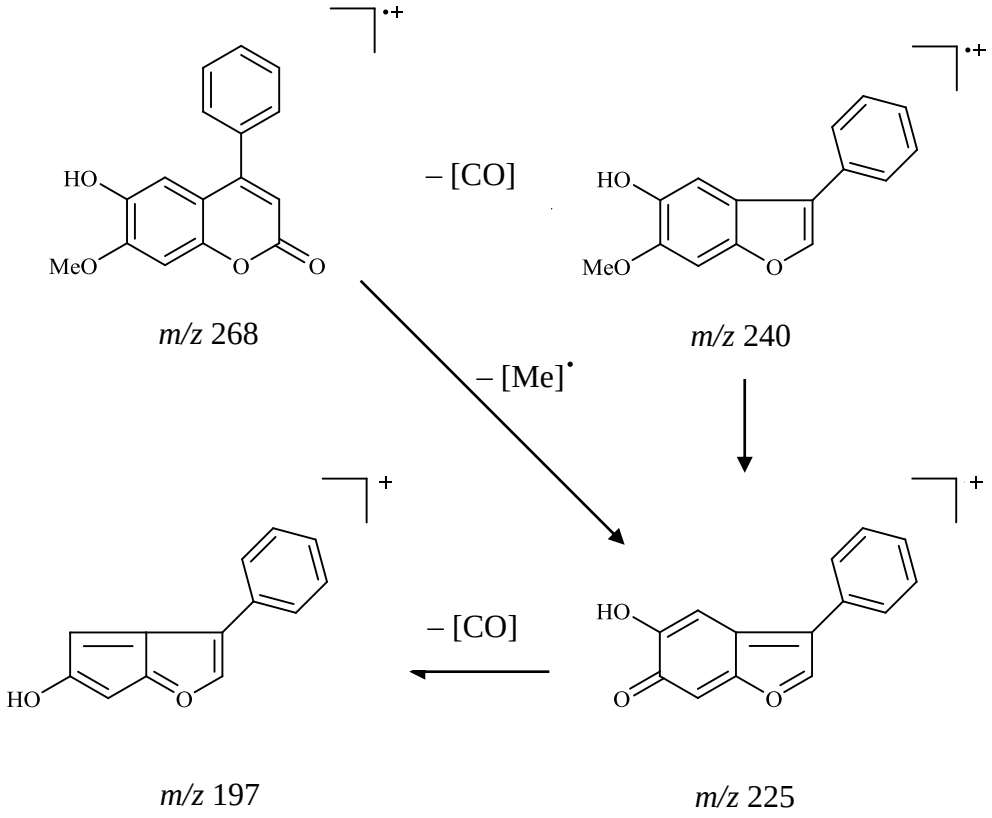


(iii) (iv)

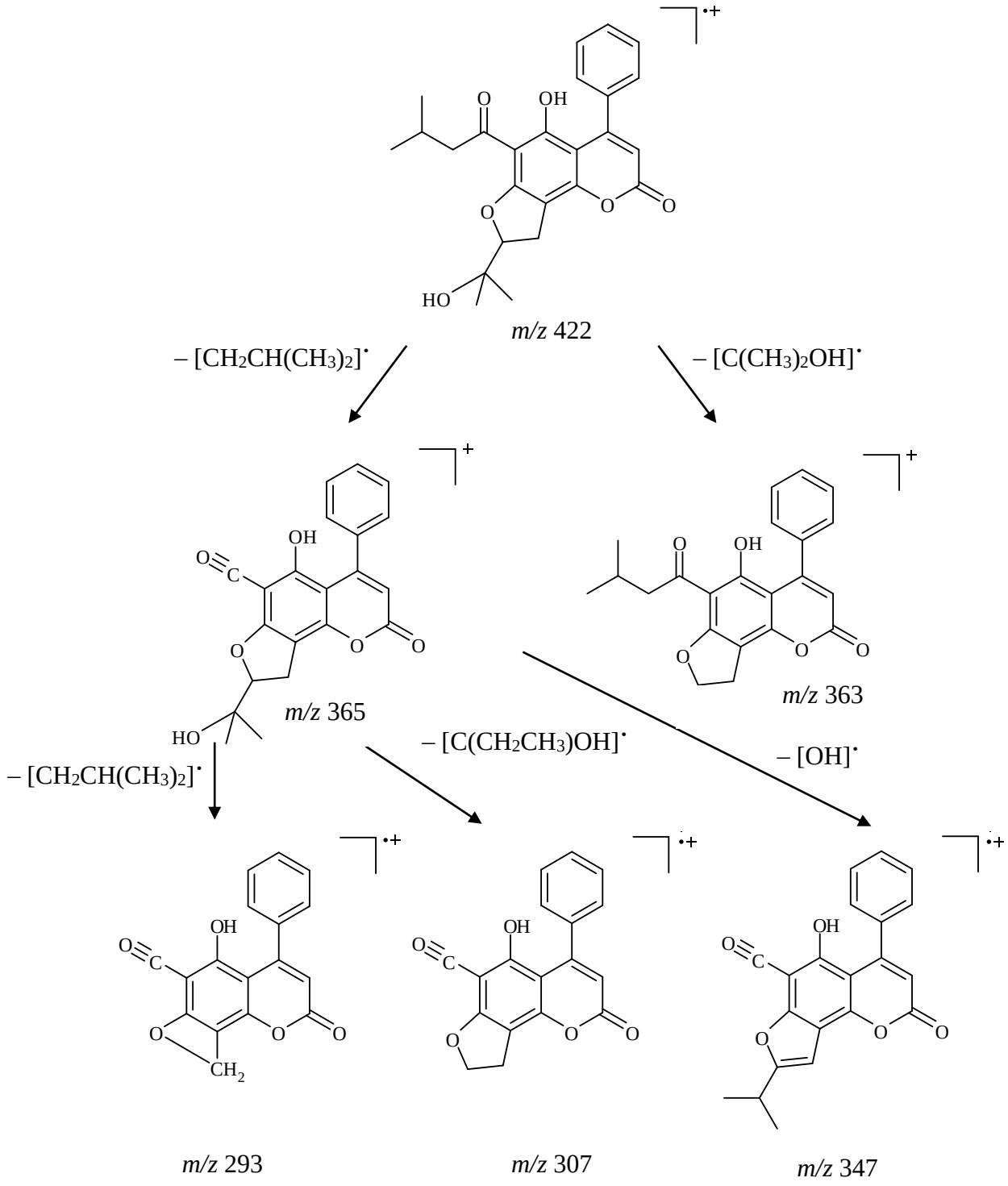
Figure 2.2: Typical UV Spectrum of (iii) 6-Acyl-5,7-dihydroxy-8-prenylcoumarin and (iv) 8-Acyl-5,7-dihydroxy-6-prenylcoumarin in Ethanol

2.4.2 Mass Spectrometry of Coumarins

The mass spectra of the 4-phenylcoumarins show the loss of CO from the molecular ion resulting in a benzofuran cation. However, the lower mass fragments are not intense. The fragmentation pathway is demonstrated for dalbergin (Scheme 2.6). The fragmentation pathway is demonstrated for dalbergin (Scheme 2.6). The fragmentations of the coumarins are mainly due to the loss of side chains at C-6 and C-8. The acyl group displays the loss of the group attached to it (Scheme 2.7)[80].



Scheme 2.6: Mass Spectral Fragmentation Pathway for Dalbergin[80]



Scheme 2.7: Mass Spectral Fragmentation Pathway of a 4-Phenylcoumarin[80]

2.4.3 ^{1}H NMR Spectroscopy of 4-Phenylcoumarins

The ^{1}H NMR spectrum can yield valuable and important information leading to the structural elucidation of coumarins. The chemical shifts are very dependent on the position of the protons and the substituents with respect to the coumarin nucleus. Several general features have been observed in the proton shifts of these coumarins. Figure 2.3 shows the structure of a 4-phenylcoumarin[81].

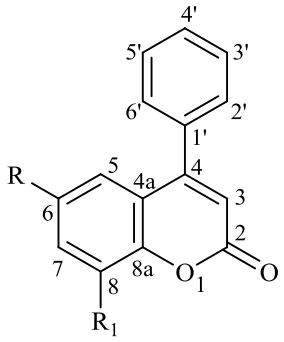


Figure 2.3: The Structure of a 4-Phenylcoumarin

There are some characteristic features for the chemical shifts of the 4-phenylcoumarins. When position C-4 is substituted by a phenyl group, two sets of multiplets will be apparent in the ^{1}H NMR spectrum, at δ 7.30 – 7.40 and δ 7.41 – 7.56 (integrated for two and three protons, respectively), along with a characteristic H-3 singlet at δ 5.95 – 6.02[46, 84]. For a prenyl substituent at position C-6 or C-8, a broad triplet can be detected at δ 5.07 - 5.21, with coupling constant of 6.0 – 7.5 Hz, and a doublet for the methylene protons at δ 3.30 – 3.57 (J = 6.5 - 7.5 Hz)[46, 76, 84]. In addition, for a geranyl substituent at position C-6 or C-8, two broad triplets can be detected at δ 4.95 - 5.22, with coupling constants of 6.0 – 7.5 Hz, a doublet for the methylene protons at δ 3.30 – 3.57 (J = 6.5 - 7.5 Hz), multiplets for two more methylene protons at δ 1.98 – 2.20 and three methyl singlets at δ 1.52 – 1.70[46].

Positions C-5 and C-7 are commonly substituted by hydroxyl and methoxyl groups. In the case of 6-acyl-5,7-dihydroxycoumarin derivatives, both of the hydroxyl protons will be chelated due to the presence of the acyl carbonyl at C-6, and they will appear at around δ 10.30 – 12.20 as two singlets. However, in the case of the 8-acyl-5,7-dihydroxycoumarin derivatives, the hydroxyl proton at C-7 will appear further downfield at δ 14.30 – 14.55 than the C-5 hydroxyl proton, which appears at δ 5.58 – 6.00. A rapid differentiation between 6-acyl-5,7-dihydroxycoumarin and 8-acyl-5,7-dihydroxycoumarin derivatives is that in 8-acyl-5,7-dihydroxycoumarin, only the C-7 hydroxyl group is chelated by the C-8 acyl group. However, in 6-acyl-5,7-dihydroxycoumarin, both the C-5 and C-7 hydroxyl groups are chelated by the C-6 acyl group[46, 84].

Common substituents of the acyl group are 3-methylbutyryl, 2-methylbutyryl or 2-methylpropionyl. For a 3-methylbutyryl group, a doublet integrating for six protons will appear at δ 0.90 – 1.10, with J = 6.5 – 7.0 Hz, in addition to a methylene doublet at δ 3.00 – 3.30 (J = 6.5 – 7.0 Hz) and a multiplet at δ 2.10 – 2.25 (integrating for one proton). For a 2-methylbutyryl group, two methyl protons will be visible as a doublet and a triplet at δ 1.00 – 1.30 (J = 6.5 – 7.0 Hz) and δ 0.80 – 1.10 (J = 7.0 – 7.5 Hz). As for the 2-methylpropionyl group, the two methyl groups will appear as a six-proton doublet at δ 1.25 – 1.30 (J = 6.5 – 7.0 Hz), together with a multiplet for one proton at δ 3.98 – 4.10. This multiplet is more downfileld than that of the 2-methylbutyryl due to its position nearer to the acyl carbonyl[46-47, 84-85].

2.4.4 ^{13}C NMR Spectroscopy of 4-Phenylcoumarins

The ^{13}C chemical shifts of coumarin were assigned by comparison with the spectrum of cinnamic acid, and from the knowledge that for benzenoid methine carbons introduction of an acyloxy function into the benzene ring causes small shifts of the *meta* carbons, and shifts the signals of the *para* carbons to higher field, while the *ortho* carbons become even more shielded (Figure 2.4)[86].

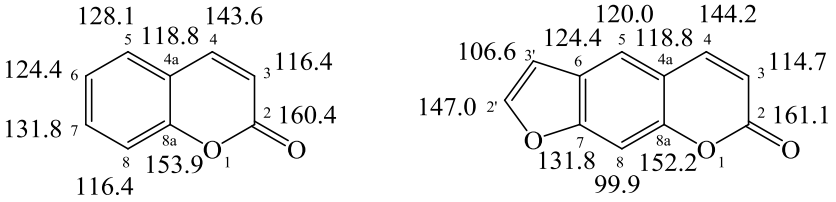


Figure 2.4: Structures and ^{13}C Chemical Shifts (δ) of Coumarin and a Furanocoumarin, Psoralen

Recording the ^{13}C NMR spectra of coumarins in different solvents, such as deuterochloroform, dimethyl sulfoxide or methanol, shows that the shifts change only by about ± 1 ppm. Using titanium tetrachloride as a complexing reagent causes strong downfield shifts for the signals of C=O and C-4, and much less deshielding for C-4a, C-5, C-6, C-7 and C-8. The resonances of C-3 and C-8a are shifted to higher field. An enhanced contribution of a dipolar mesomeric form is thought to be responsible for this effect[86].

Aromatic carbon (sp^2) atoms, as in 4-phenyl coumarins, absorb at δ 128.5 in $CDCl_3$ (deuterated chloroform) or CCl_4 (carbon tetrachloride). Shifts of the aromatic carbon atom directly attached to a substituent are correlated with substituent's electronegativity after correcting for magnetic anisotropy effects. For example, an aromatic carbon atom attached to an ethylene resonates at δ 137.6, while that attached to a methoxy resonates at δ 159.9[87].

CHAPTER 3

CHEMICAL CONSTITUENTS FROM *M. elegans* AND *M. kunstleri*

An attempt at the bioassay-guided isolation of the acetylcholinesterase (AChE) and neuroprotective compounds was performed on the bark material of *Mesua elegans* and *Mesua kunstleri*. In addition, a thorough phytochemical analysis was carried out on the active hexane extracts of these plants. The present chapter deals with phytochemical studies of the bark of *M. elegans* and *M. kunstleri*.

Various chromatographic techniques, such as column chromatography (CC), preparative thin layer chromatography (PTLC), high performance liquid chromatography (HPLC) and centrifugal chromatographic technique (CPC) were used in this study to isolate the compounds. The structures were determined by using combinations of different spectroscopic methods; 1D NMR (^{1}H, ^{13}C, DEPT), 2D NMR (COSY, NOESY, HSQC, HMBC and NOESY), UV, IR and HRESIMS. The structures of the compounds were also elucidated by literature comparison with previous studies.

3.1 Chemical Constituents from the Hexane Extracts of *M. elegans* and *M. kunstleri*

Isolation from the hexane extracts of the bark of *M. elegans* and *M. kunstleri* yielded twenty-four 4-phenylcoumarins, and Table 3.1 shows the list of compounds isolated from these plants. A total of twenty-two compounds were isolated from *M. elegans*, of which thirteen are new; mesuagenin A **189**, mesuagenin B **190**, mesuagenin C **191**, mesuagenin F **195**, mesuagenin D **196**, mesuagenin G **198**, mesuagenin H **199**, mesuagenin I **200**, mesuagenin J **201**, mesuagenin K **202**, mesuagenin L **203**,

mesuagenin M **204** and mesuagenin N **205**. From *M. kunstleri*, a total of ten compounds were isolated, of which one is new; mesuagenin E **188**.

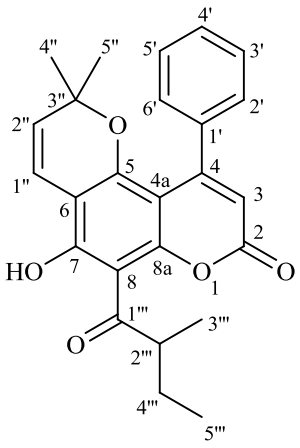
The structures of these 4-phenylcoumarins were identified through the analysis of their spectral data, and also by comparison of their spectral data with those described in the literature. The following sub-chapters discuss briefly the structural elucidation of the isolated compounds.

Table 3.1: Chemical Constituents from *M. elegans* and *M. kunstleri*

Plants	**Isolates**	**Name of Isolates**
M. elegans	Compound B	Mammea A/BA cyclo D or isomammeigin **187**
	Compound D	Mesuagenin A **189**
	Compound E	Mesuagenin B **190**
	Compound F	Mammea A/BB or isomammeisin **55**
	Compound G	Mammea A/BA **28**
	Compound H	Mesuagenin C **191**
	Compound I	5,7-dihydroxy-8-(2-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one **47**
	Compound J	5,7-dihydroxy-8-(3-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one **48**
	Compound K	Ochrocarpin E **192**
	Compound L	Mammea A/BB cyclo F **193**
	Compound M	Mammea A/BA cyclo F **194**
	Compound N	Mesuagenin F **195**
	Compound O	Mesuagenin D **196**
	Compound P	Isodisparfuran **197**
	Compound Q	Mesuagenin G **198**
	Compound R	Mesuagenin H **199**

Plants	**Isolates**	**Name of Isolates**
	Compound S	Mesuagenin I **200**
	Compound T	Mesuagenin J **201**
	Compound U	Mesuagenin K **202**
	Compound V	Mesuagenin L **203**
	Compound W	Mesuagenin M **204**
	Compound X	Mesuagenin N **205**
M. kunstleri	Compound A	Mammea A/BB cyclo D or ponnalide **186**
	Compound B	Mammea A/BA cyclo D or isomammeigin **187**
	Compound C	Mesuagenin E **188**
	Compound D	Mesuagenin A **189**
	Compound E	Mesuagenin B **190**
	Compound F	Mammea A/BB or isomammeisin **55**
	Compound G	Mammea A/BA **28**
	Compound H	Mesuagenin C **191**
	Compound I	5,7-dihydroxy-8-(2-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one **47**
	Compound J	5,7-dihydroxy-8-(3-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one **48**

3.1.1 Compound A: Mammea A/BB cyclo D or Ponnalide 186



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Compound A was isolated as a pale yellowish oil, and the HRESIMS spectrum showed an $[M+Na]^+$ ion at *m/z* 427.1510 (calculated 427.1521), which corresponded to the molecular formula $C_{25}H_{24}O_5$. The UV spectrum (EtOH) supported an 8-acyl-5,7-dioxycoumarin type, with absorptions at λ_{max} 237, 284 and 339 nm[82]. The IR spectrum showed absortions at ν_{max} 1740 cm^{-1} which belongs to an α-pyrone and a broad peak at 3461 cm^{-1} due to OH stretching[47, 88].

The ^1H NMR spectrum of compound A showed signals typical of a 4-phenylcoumarin. A typical H-3 singlet was observed at δ 6.01 in the ^1H NMR spectrum, characteristic of the C-3 proton of a 4-substituted coumarin. The presence of a mono-substituted phenyl group at C-4 was corroborated by the presence of two sets of multiplets, centred at δ 7.39 (H-3'-H-5') and 7.26 (H-2', H-6'), corresponding to three and two aromatic protons, respectively. Furthermore, the ^1H NMR spectrum of compound A supported the presence of a chelated hydroxyl by revealing a proton singlet characteristic of a chelated hydroxyl at δ 14.62, and a broad OH absorption depicted from the IR spectrum further supported this finding. All these observations from the ^1H NMR spectrum, together with

the UV and IR data reported earlier, suggested the presence of an 8-acyl-5,7-dioxy-4-phenylcoumarin type skeleton[47].

The ^{13}C NMR spectrum of compound A showed four methyl, one methylene, nine methine and eleven quaternary signals. The ^{1}H NMR spectrum also showed two singlets at δ 0.95 (H-4") and 0.96 (H-5"), integrating for three protons each, along with two doublets of one proton each at δ 6.62 (*J* = 10.1 Hz, H-1") and 5.38 (*J* = 10.1 Hz, H-2"). The olefinic protons H-1" and H-2" correlated with the quaternary carbon C-6 (δ 106.00) in the HMBC spectrum. All these observations indicated the presence of a cyclised prenyl group in compound A, substituted at position C-6.

The signals of two methylene protons as two sets of multiplets at δ 1.50 (1H, *m*, H-4"'α) and 1.92 (1H, *m*, H-4"'β) were apparent in the ^{1}H NMR spectrum. In the HMBC spectrum, these protons correlated with a carbonyl (C-1"', δ 210.67), a methine (C-2"', δ 47.09) and two methyl groups (C-3"', δ 16.72 and C-5"', δ 11.90). Protons of the two methyls (C-3"" and C-5"') were observed as a doublet at δ 1.28 (*J* = 6.7 Hz, H-3"') and as a triplet at δ 1.02 (*J* = 7.3, H-5"'), respectively. These elements confirmed the presence of the 2-methylbutanoyl moiety linked at C-8.

The positions of the substituents were confirmed by spectroscopic analysis and also by direct comparison with the literature values. Thus, compound A was identified as the known mammea A/BB cyclo D or ponnalide **186**, which was first isolated from *Calophyllum inophyllum*[89,90], a plant from the same family as *M. elegans* and *M. kunstleri*.

Table 3.2: ^{1}H NMR, ^{13}C NMR and COSY (in $CDCl_3$, 400 MHz) of Compound A **186**

Position	δ_H, *J* (Hz)	δ_C	COSY
2	-	159.0	
3	6.01 (1H, *s*)	112.1	
4	-	157.1	
4a	-	102.3	
5	-	156.4	
6	-	106.0	
7-OH	14.62 (1H, *s*)	164.0	
8	-	104.3	
8a	-	156.2	
1'	-	140.2	
2'	7.26 (1H, *m*, Ar)	127.3	
3'	7.39 (3H, *m*, Ar)	127.8	
4'		127.9	
5'		127.8	
6'	7.26 (1H, *m*, Ar)	127.3	
1"	6.62 (1H, *d*, *J* = 10.1)	115.5	2"
2"	5.38 (1H, *d*, *J* = 10.1)	126.9	
3"	-	79.2	
4"	0.95 (3H, *s*)	27.9	
5"	0.96 (3H, *s*)	27.9	
1'"	-	210.7	
2'"	3.95 (1H, *m*)	47.1	3'"
3'"	1.28 (3H, *d*, *J* = 6.7)	16.7	
4'"α	1.50 (1H, *m*)	27.3	4'"β, 5'"
4'"β	1.92 (1H, *m*)		4'"α, 5'"
5'"	1.02 (3H, *t*, *J* = 7.3)	11.9	

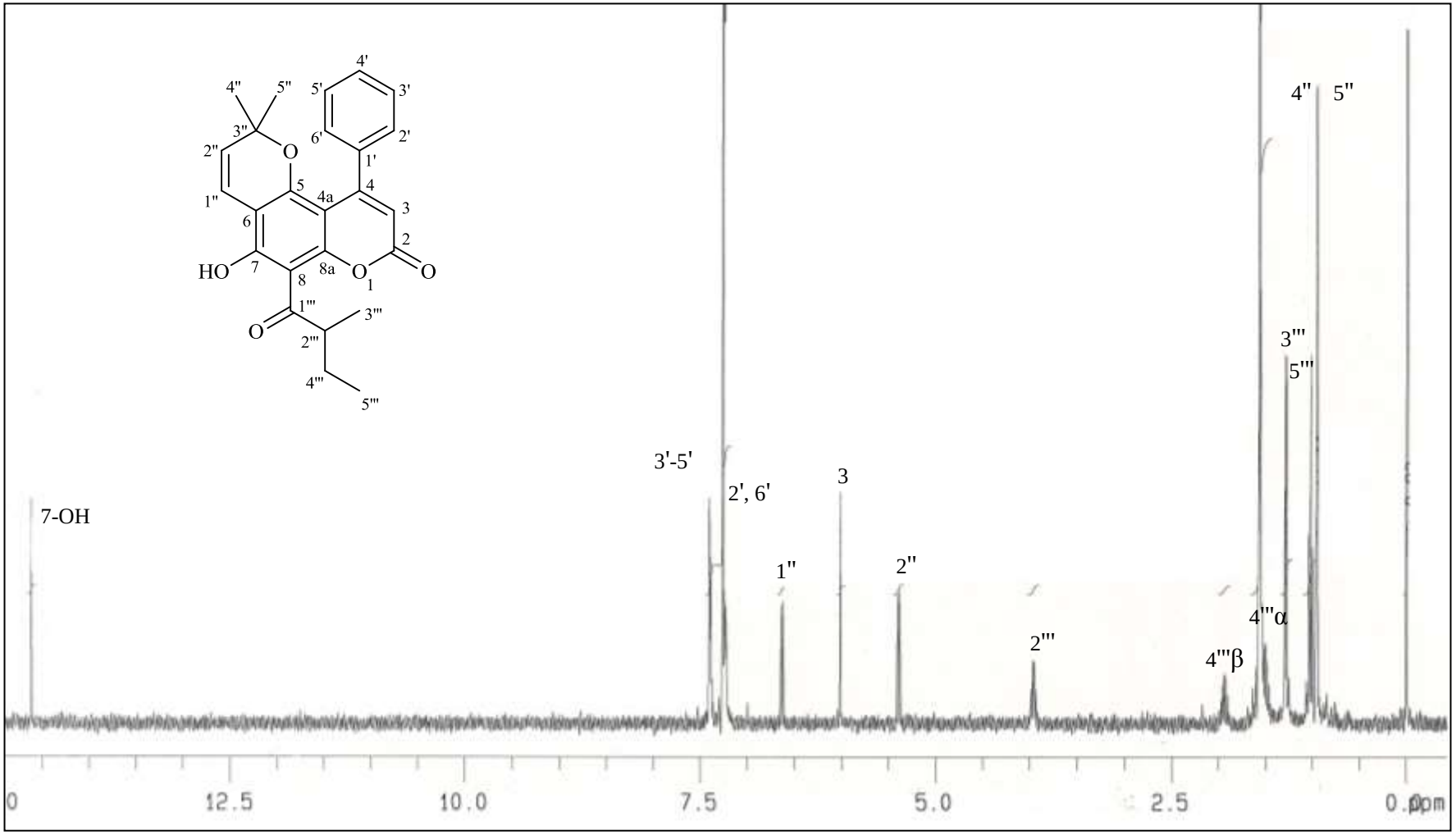
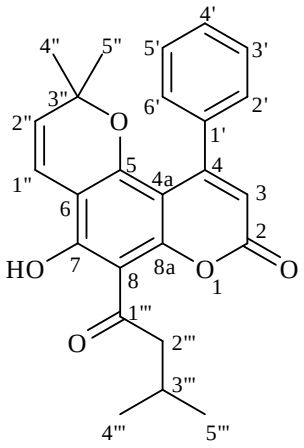


Figure 3.1: ^{1}H NMR Spectrum of Compound A **186**

3.1.2 Compound B: Mammea A/BA cyclo D or Isomameigin 187



187

A pale yellowish oil was obtained as compound B. The HRESIMS revealed an $[M+Na]^+$ ion at *m/z* 427.1535 (calculated 427.1521), which corresponded to the molecular formula $C_{25}H_{24}O_5$. The IR spectrum showed absortions at ν_{max} 1382 (geminal dimethyl), 1740 (α-pyrone) and a broad peak at 3461 cm^{-1} (OH stretching)[47, 88]. The UV spectrum (EtOH) supported an 8-acyl-5,7-dioxycoumarin type, with absorptions at λ_{max} 237, 284 and 339 nm[82].

The ^1H and ^{13}C NMR spectra of compound B are reminiscent of those of compound A, which suggested a close structural relationship between these two compounds. Indeed, the same substituents for the coumarin could be characterized in both compounds at positions C-4, C-5, C-6 and C-7, namely, a typical H-3 signal of a 4-phenylcoumarin (δ 6.00, *s*, H-3), a monosubstituted phenyl (δ 7.37 and 7.23, 5H, *m*, H-2'-H6'), a cyclized prenyl group [δ 6.60 (1H, *d*, *J* = 10.1 Hz, H-1"), 5.37 (1H, *d*, *J* = 10.1 Hz, H-2"), 0.94 (6H, *s*, H-4" and H-5") and a chelated hydroxyl group (δ 14.63, *s*, 7-OH)[47].

However in the ^1H NMR spectrum, compounds A and B exhibited quite different patterns for the remaining substituent at C-8. The cross correlation deduced from COSY

spectrum revealed the presence of an *iso*-butyryl spin system with signals at δ 3.17 (2H, *d*, *J* = 6.7 Hz, H-2'''), 2.31 (1H, *m*, H-3''') and 1.04 (6H, *d*, *J* = 6.7, H-4''' and H-5'''). In the HMBC spectrum of compound B, the methylene protons and the methine proton produced cross-peaks with a keto function at δ 206.2, which corresponded to C-1'''. With this, it was identified that a 3-methylbutanoyl chain was the other substituent of the 4-phenylcoumarin, instead of a 2-methylbutanoyl chain as in compound A.

The assignments of all the proton and carbon signals of compound B were confirmed by direct comparison with the literature values. Thus, compound B was identified as mammea A/BA cyclo D or isomammeigin **187**, which was first isolated from *Kilmeyera pumila* Pohl (Clusieaceae)[91].

Table 3.3: ^{1}H NMR, ^{13}C NMR and COSY (in $CDCl_3$, 400 MHz) of Compound B **187**

Position	δ_H, *J* (Hz)	δ_C	COSY
2	-	159.0	
3	6.00 (1H, *s*)	112.1	
4	-	157.1	
4a	-	102.3	
5	-	156.4	
6	-	106.0	
7-OH	14.63 (1H, *s*)	163.9	
8	-	104.3	
8a	-	156.2	
1'	-	140.2	
2'	7.23 (1H, *m*, Ar)	127.3	
3'	7.37 (3H, *m*, Ar)	127.8	
4'		127.9	
5'		127.8	
6'	7.23 (1H, *m*, Ar)	127.3	
1"	6.60 (1H, *d*, *J* = 10.1)	115.5	2"
2"	5.37 (1H, *d*, *J* = 10.1)	126.9	
3"	-	79.2	
4"	0.94 (6H, *s*)	27.6	
5"		27.6	
1'"	-	206.2	
2'"	3.17 (2H, *d*, *J* = 6.7)	53.7	3'"
3'"	2.31 (1H, *m*)	25.8	4'", 5'"
4'"	1.04 (6H, *d*, *J* = 6.7)	22.9	
5'"		22.9	

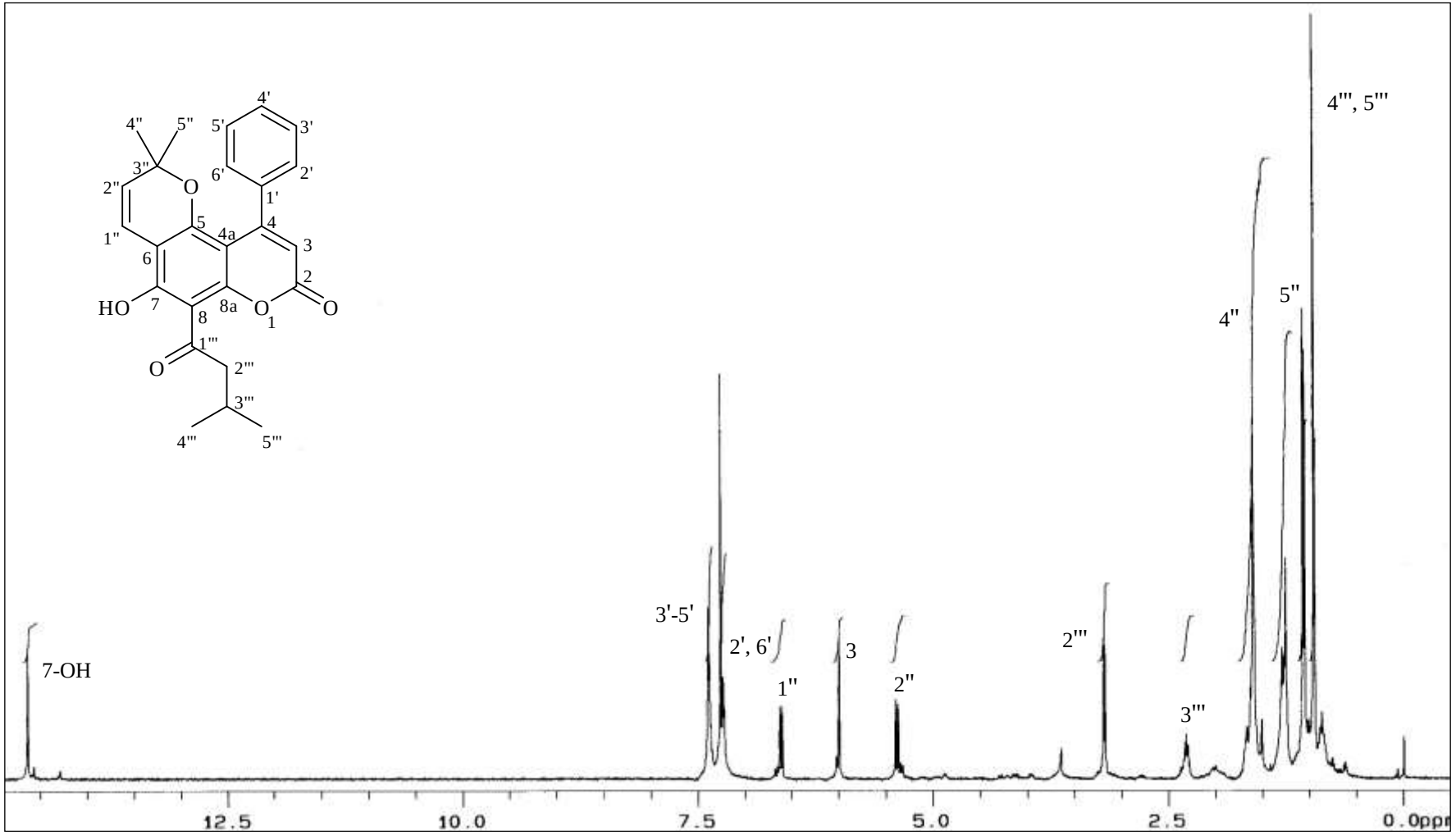
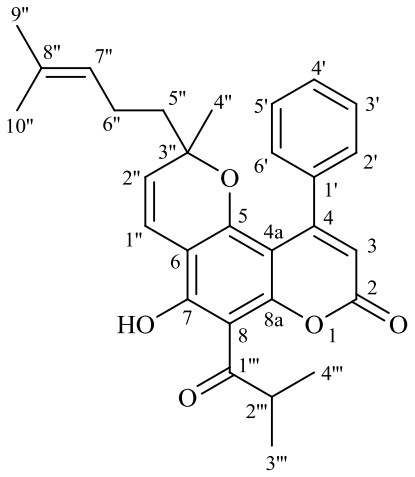


Figure 3.2: ^{1}H NMR Spectrum of Compound B **187**

3.1.3 Compound C: Mesuagenin E 188



188

Separation by using column chromatography, followed by HPLC, afforded compound C as a yellowish oil. The molecular formula of $C_{29}H_{30}O_5$ was deduced from the HRESIMS measurement of compound C; $[M+Na]^+$ ion at *m/z* 481.1553 (calculated 481.1991). The UV spectrum (EtOH) supported an 8-acyl-5,7-dihydroxycoumarin type, with absorptions at λ_{max} 237, 285 and 335 nm[82]. The IR spectrum showed absorptions at υ_{max} 1382 cm^{-1} which belongs to a geminal dimethyl, and at 1612 cm^{-1} which belongs to a chelated acyl group. The IR spectrum also exhibited a strong peak at 1741 cm^{-1} due to the presence of an α-pyrone and a broad peak at 3461 cm^{-1} due to OH stretching[47, 88].

In the ^1H NMR spectrum, the typical H-3 singlet of a 4-phenylcoumarin moiety was observed at δ 6.03. The presence of a mono-substituted phenyl group at C-4 was corroborated by the presence two sets of multiplets, centred at δ 7.41 (H-3'-H-5') and δ 7.24 (H-2', H-6'), corresponding to three and two aromatic protons, respectively. In addition, a proton singlet characteristic of a chelated hydroxyl at δ 14.60 (7-OH) was also observed. All these observations supported the presence of an 8-acyl-5,7-dioxy-4-phenylcoumarin type[47].

^{13}C NMR spectrum of compound C showed the presence twenty-nine signals; five methyls, two methylenes, ten methines and twelve quaternaries. Furthermore, the ^{1}H NMR spectrum also showed a set of doublets, most probably belonging to the olefinic protons H-1" and H-2" at δ 6.71 (*J* = 10.1 Hz, H-1") and 5.36 (*J* = 10.1 Hz, H-2"). A methyl singlet was also apparent at δ 1.00 (H-4"). All these protons correlated with the quaternary carbon C-3" (δ 81.9) in the HMBC spectrum. In addition, the olefinic protons (H-1" and H-2") correlated with the quaternary C-6 (δ 105.6). This inferred the presence of a cyclised prenyl group attached to C-6. However, the methyl C-5" signal as a singlet was absent, replaced by a methylene multiplet at δ 1.21 (H-5"). In the HMBC spectrum, δ 1.67 (2H, *m*, H-6") correlated with the methylene carbon (C-5", δ 40.9), an sp^3 methine (C-7", δ 123.5) and an sp^2 quaternary carbon (C-8", δ 131.8). C-8", in return, correlated with two geminal dimethyl protons (δ 1.54, H-9" and δ 1.67, H-10") in the HMBC spectrum. These resonances implied the presence of another prenyl group attached to C-5". All this information established the nature of the C-5/C-6 substituent as the 3-methyl-3-prenyl-Δ^3-pyran ring system.

The cross correlations deduced from the COSY spectrum revealed the presence of an *iso*-propyl spin system, with signals at δ 4.14 (1H, *m*, H-2"') and δ 1.32 (6H, *d*, *J* = 6.7 Hz, H-3"' and H-4"'). In the HMBC spectrum, the methine proton and the methyl protons gave cross-peaks with the C-1"' keto function at δ 210.7. With this, it was identified that an *iso*-butanoyl chain was the other substituent on the 4-phenylcoumarin nucleus, which was attached at C-8.

In view of the above data, and a thorough search in the SciFinder database, it was deduced that compound C was a new compound with the proposed structure of mesuagenin E **188**.

Table 3.4: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound C **188**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	158.9		
3	6.03 (1H, *s*)	111.9		2, 4a, 1'
4	-	156.8		
4a	-	102.0		
5	-	156.6		
6	-	105.6		
7-OH	14.60 (1H, *s*)	163.9		7,8
8	-	103.2		
8a	-	156.2		
1'	-	140.1		
2'	7.24 (1H, *m*, Ar)	127.0		4
3'	7.41 (3H, *m*, Ar)	127.7		1', 2'
4'		127.8		
5'		127.7		
6'	7.24 (1H, *m*, Ar)	127.1		
1"	6.71 (1H, *d*, *J* = 10.1)	116.0	2"	6, 7, 3"
2"	5.36 (1H, *d*, *J* = 10.1)	125.4	1"	6, 3", 5"
3"	-	81.9		
4"	1.00 (3H, *s*)	26.8		2", 3", 5"
5"	1.21 (2H, *m*)	40.9		6"
6"	1.67 (2H, *m*)	22.8		3", 5", 7", 8"
7"	4.91 (1H, br*t*, *J* = 6.8)	123.5	6", 9"	5", 6", 9", 10"
8"	-	131.8		
9"	1.54 (3H, *s*)	17.7		7", 8", 10"
10"	1.67 (3H, *s*)	25.6		7", 8", 9"
1'"	-	210.7		
2'"	4.14 (1H, *m*)	40.3	3'", 4'"	1'", 3'", 4'"
3'"	1.32 (6H, *d*, *J* = 6.7)	19.3	2'", 4'"	1'", 2'", 4'"
4'"		19.3	2'", 3'"	1'", 2'", 3'"

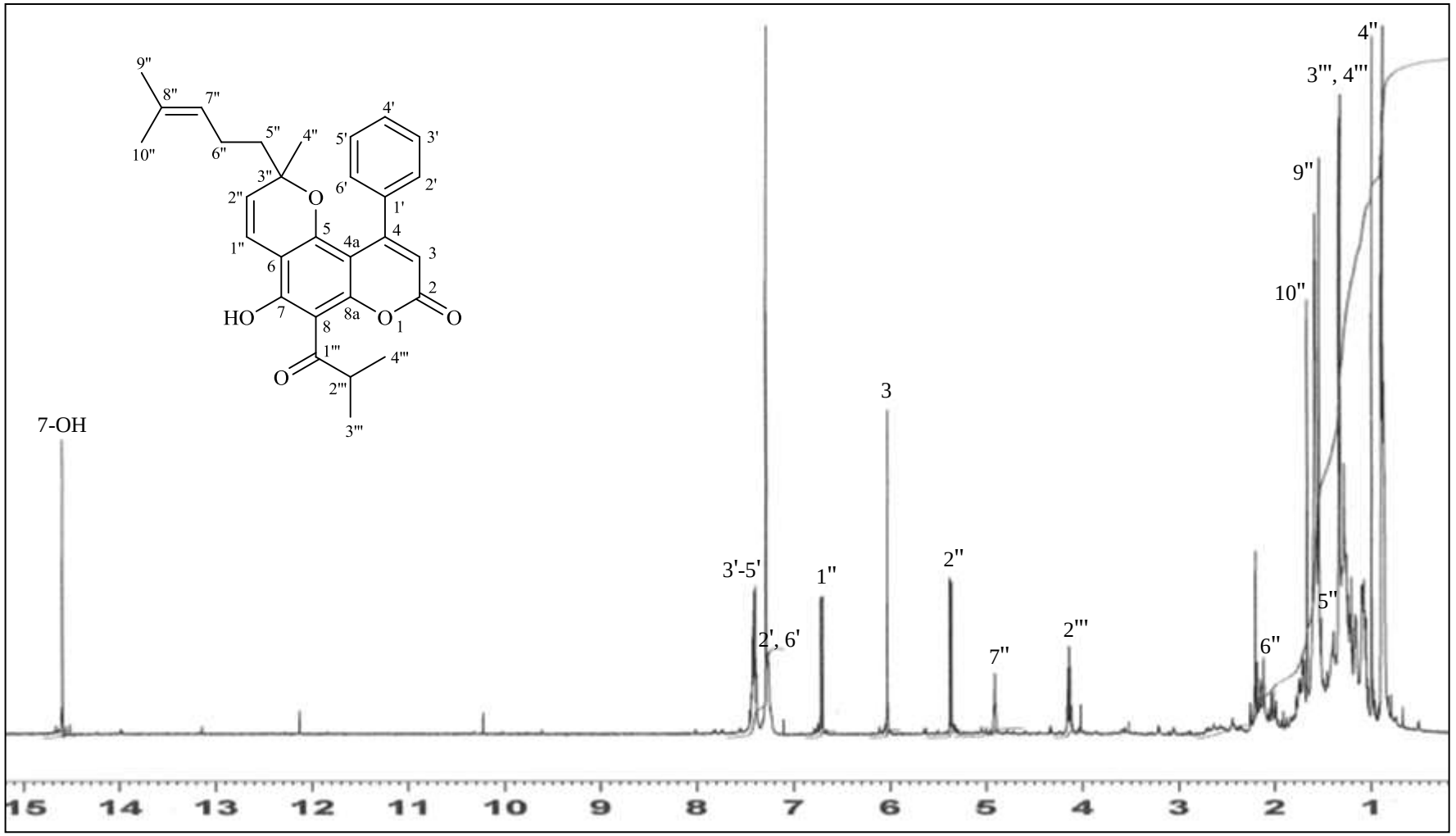


Figure 3.3: ^{1}H NMR Spectrum of Compound C **188**

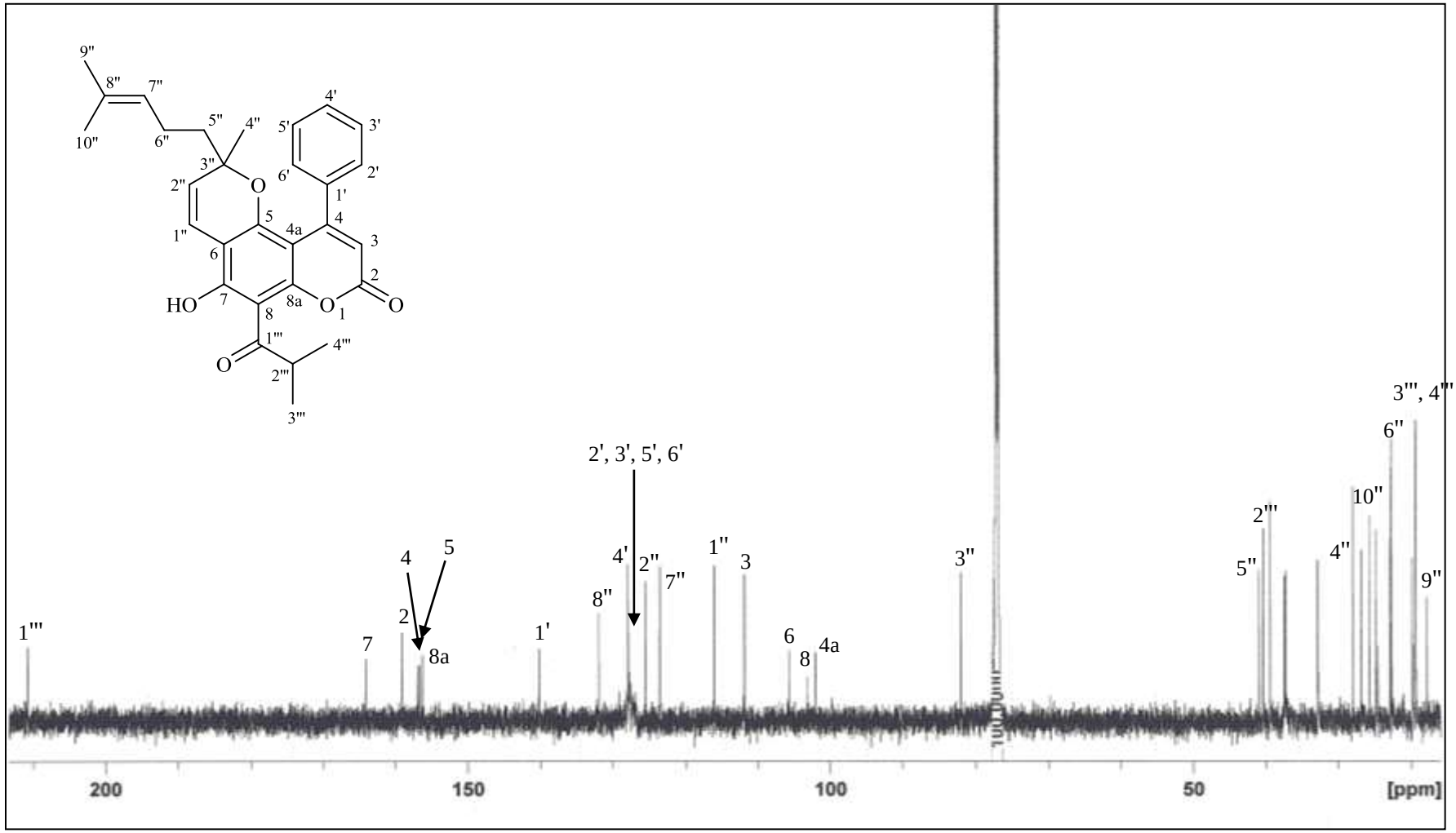
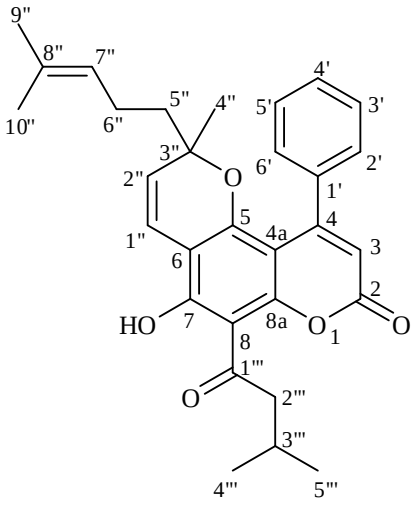


Figure 3.4: ^{13}C NMR Spectrum of Compound C **188**

3.1.4 Compound D: Mesuagenin A 189



189

The molecular formula of compound D was determined by the HRESIMS measurement of its $[M+Na]^+$ ion at *m/z* 495.2128 (calculated 495.2147), as $C_{30}H_{32}O_5$. Compound D was separated as a yellowish oil with $[\alpha]^{26}_D$ +10 (*c* 0.0002, EtOH). The IR spectrum also exhibited strong peaks at 1382 (geminal dimethyl), 1612 (chelated acyl group), 1741 (α-pyrone) and a broad peak at 3461 cm^{-1} (OH stretching)[47, 88]. The UV spectrum (EtOH) supported an 8-acyl-5,7-dioxycoumarin type, with absorptions at λ_{max} 272 and 313 nm[82].

The ^{13}C NMR spectrum of compound D displayed a total of thirty carbon signals; five methyls, three methylenes, ten methines and twelve quaternary carbons. The ^1H NMR spectrum of compound D showed striking similarities with the ^1H NMR spectrum of compound C, with similar substituents observed at positions C-3 (the typical H-3 singlet of a 4-substituted coumarin at δ 5.99), C-4 (monosubstituted phenyl; δ 7.38 and 7.22, 5H, *m*, H-2'-H6'), C-5/C-6 [3-methyl-3-prenyl-Δ^3-pyran ring; δ 6.65 (1H, *d*, *J* = 10.4 Hz, H-1"), 5.32 (1H, *d*, *J* = 10.4 Hz, H-2"), 0.96 (3H, *s*, H-4"), 1.21 (2H, *m*, H-5"), 1.67 (2H, *m*, H-6"), 1.50 (3H, *s*, H-9") and 1.63 (3H, *s*, H-10")] and C-7 (chelated hydroxyl group; δ 14.64, *s*, 7-OH).

Nonetheless, compound D exhibited notably different patterns for the remaining substituent at C-8 in the ^{1}H NMR spectrum as compared with compound C; δ 3.16 (2H, *d*, *J* = 6.7 Hz, H-2'''), 2.31 (1H, *m*, H-3''') and δ 1.04 (6H, *d*, *J* = 6.7 Hz, H-4''' and H-5'''). An *iso*-butyryl spin system could be deduced from the COSY and HMBC correlations of compound D. With this, it was identified that a 3-methylbutanoyl chain was the other substituent of the 4-phenylcoumarin, which was attached at C-8.

After a thorough search in the SciFinder database, it was found that compound D was a new chemical entity, with the IUPAC name 7-hydroxy-6-methyl-6-(4-methylpent-3-enyl)-8-(3-methylbutanoyl)-4-phenyl-2*H*-pyrano[2,3-*h*]chromen-2-one or which has been named as mesuagenin A[92] **189**.

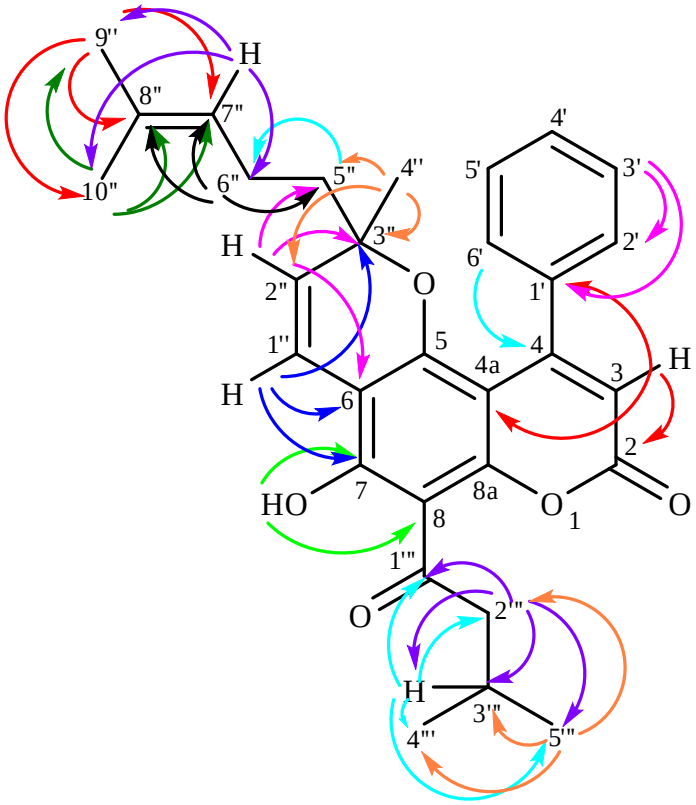


Figure 3.5: The HMBC Correlations of Compound D **189**

Table 3.5: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound D **189**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	158.8		
3	5.99 (1H, *s*)	111.9		2, 4a, 1'
4	-	157.0		
4a	-	102.0		
5	-	156.6		
6	-	105.4		
7-OH	14.64 (1H, *s*)	163.7		7,8
8	-	104.0		
8a	-	156.0		
1'	-	140.0		
2'	7.22 (1H, *m*, Ar)	127.1		4
3'	7.38 (3H, *m*, Ar)	127.6		1', 2'
4'		127.8		
5'		127.6		
6'	7.22 (1H, *m*, Ar)	127.1		
1"	6.65 (1H, *d*, *J* = 10.4)	115.9	2"	6, 7, 3"
2"	5.32 (1H, *d*, *J* = 10.4)	125.3	1"	6, 3", 5"
3"	-	81.9		
4"	0.96 (3H, *s*)	26.8		2", 3", 5"
5"	1.21 (2H, *m*)	40.9		6"
6"	1.67 (2H, *m*)	22.8		3", 5", 7", 8"
7"	4.87 (1H, br*t*, *J* = 6.7)	123.5	6", 9"	5", 6", 9", 10"
8"	-	131.8		
9"	1.50 (3H, *s*)	17.7		7", 8", 10"
10"	1.63 (3H, *s*)	25.7		7", 8", 9"
1'"	-	206.0		
2'"	3.16 (2H, *d*, *J* = 6.7)	53.5	3'"	1'", 3'", 4'", 5'"
3'"	2.31 (1H, *m*)	25.6	4'", 5'"	1'", 2'", 4'", 5'"
4'"	1.04 (6H, *d*, *J* = 6.7)	22.7		2'", 3'", 5'"
5'"		22.7		2'", 3'", 4'"

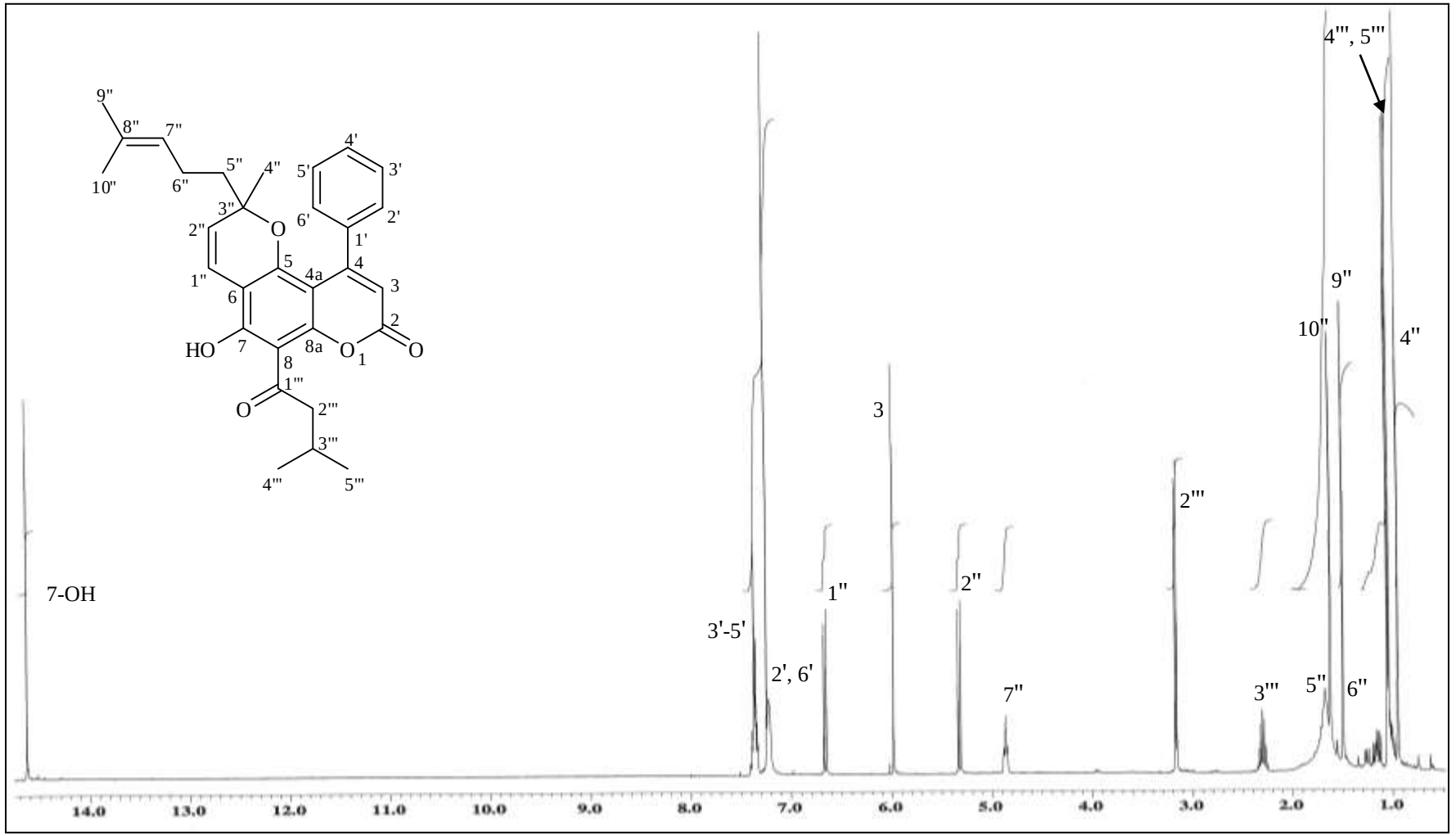


Figure 3.6: ^{1}H NMR Spectrum of Compound D **189**

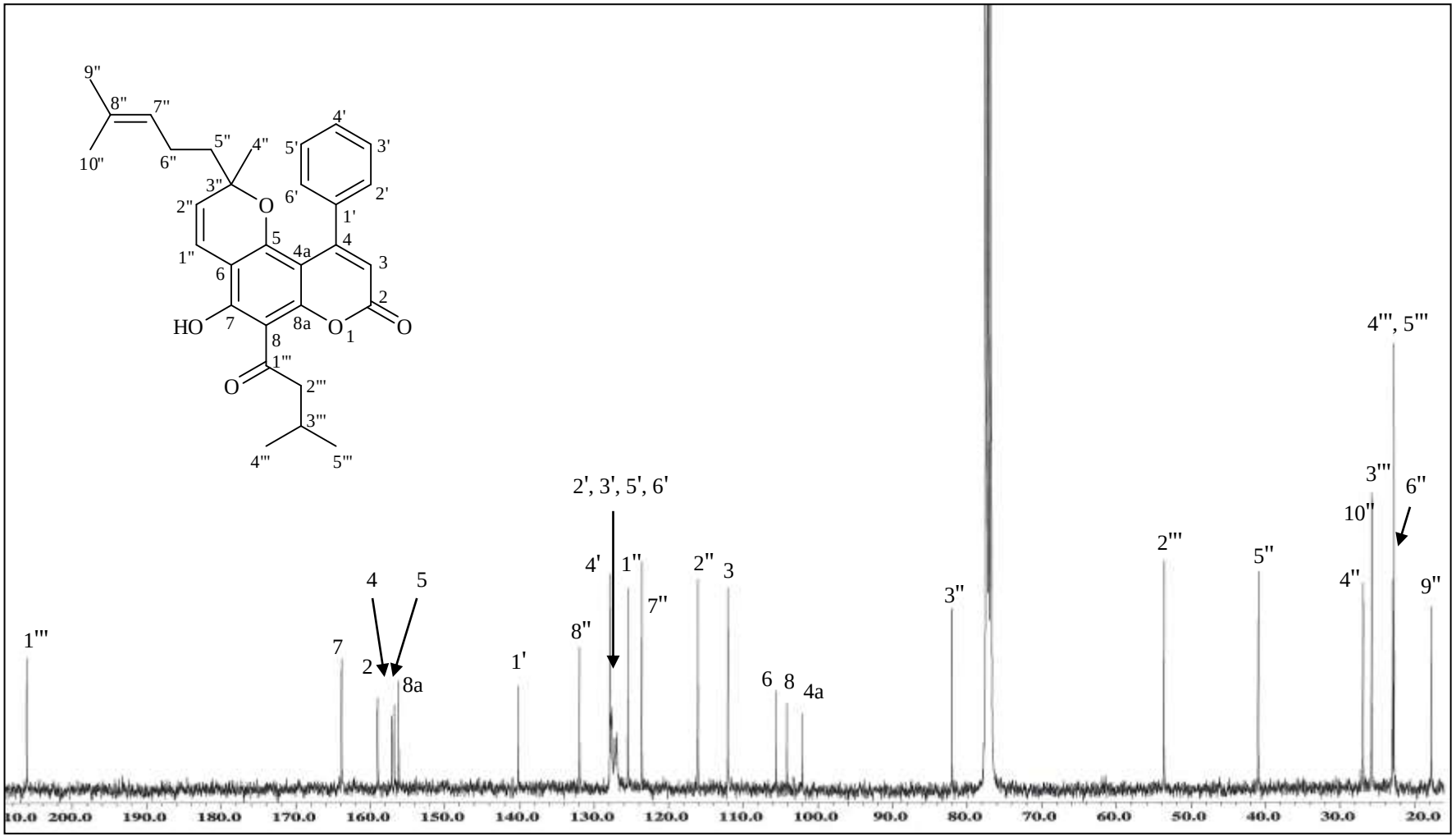
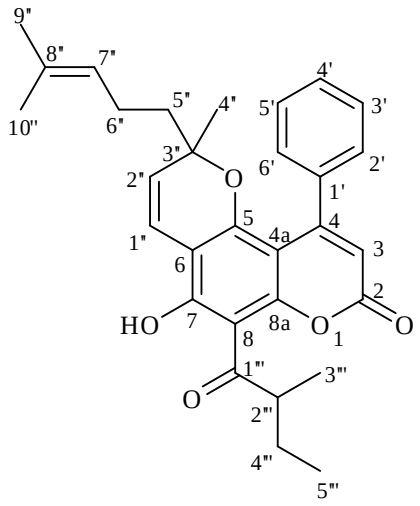


Figure 3.7: ^{13}C NMR Spectrum of Compound D **189**

3.1.5 Compound E: Mesuagenin B 190



190

Compound E was purified as a yellowish oil with $[\alpha]^{26}_{D}$ +16 (*c* 0.0003, EtOH). A pseudomolecular $[M+Na]^+$ ion at *m/z* 495.2120 (calculated 495.2147) was detected in its HRESIMS spectrum, which was associated with the molecular formula $C_{30}H_{32}O_5$. The UV spectrum showed absorptions at λ_{max} 272 and 313 nm, which supported an 8-acyl-5,7-dioxycoumarin type[82]. The IR spectrum showed strong absorptions at υ_{max} 3475 cm^{-1} (OH), 1742 cm^{-1} (α-pyrone) and 1614 cm^{-1} (chelated acyl group)[47, 88].

Similar features were evident in the ^1H and ^{13}C NMR spectra of compounds D and E, which confirmed a close structural relationship between these two compounds. The same substituents for the coumarins were characterized in these compounds through COSY, HMQC and HMBC experiments, namely a monosubstituted phenyl at C-4, a cyclized geranyl group at C-5/C-6 and a chelated hydroxyl at C-7.

However, there was an obvious difference in the ^1H NMR spectrum of compound E, in that the gem-dimethyl doublet (six protons) which was apparent in compound D was absent in this spectrum. Instead, the signal of the two methyls was replaced by a doublet

[Me-3''', δ 1.27 (*J* = 6.7 Hz)] and a triplet [Me-5''', δ 1.01 (*J* = 7.3 Hz)]. These observations indicate the presence of a 2-methylbutanoyl chain, instead of a 3-methylbutanoyl chain as in compound D, attached to the C-8 position. Therefore, indicating that compound E was indeed an isomer of compound D.

In view of the above data, and after a thorough search in the SciFinder database, it is deduced that compound E possesses the proposed structure**;** 7-hydroxy-6-methyl-6-(4-methylpent-3-enyl)-8-(2-methylbutanoyl)-4-phenyl-2*H*-pyrano[2,3-*h*]chromen-2-one, which has been synthesized by Verotta *et al*. (2004)[46]. However, this compound has been isolated as a new natural product from *M. elegans* and *M. kunstleri*, and has been named as mesuagenin B **190**.

Table 3.6: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound E **190**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	159.0		
3	5.99 (1H, *s*)	112.0		2, 4a, 1'
4	-	156.2		
4a	-	102.1		
5	-	156.6		
6	-	105.7		
7-OH	14.63 (1H, *s*)	164.0		6, 7
8	-	103.7		
8a	-	156.9		
1'	-	140.2		
2'	7.23 (1H, *m*, Ar)	127.2		4, 4'
3'	7.39 (3H, *m*, Ar)	127.6		1', 2'
4'		127.9		
5'		127.6		
6'	7.23 (1H, *m*, Ar)	127.2		
1"	6.66 (1H, *d*, *J* = 9.7)	116.1	2"	5, 7, 3"
2"	5.32 (1H, *d*, *J* = 9.7)	125.5	1"	6, 3"
3"	-	82.0		
4"	0.96 (3H, *s*)	26.9		1", 2", 5"
5"	1.20 (2H, *m*)	41.0		6"
6"	1.67 (2H, *m*)	22.9		5", 7", 8"
7"	4.87 (1H, br*t*, *J* = 6.7)	123.6		6", 9", 10"
8"	-	131.9		
9"	1.50 (3H, *s*)	17.8		7", 8", 10"
10"	1.63 (3H, *s*)	25.7		7", 8", 9"
1'"	-	210.6		
2'"	3.96 (1H, *m*)	47.0	3'"	1'", 3'", 4'", 5'"
3'"	1.27 (3H, *d*, *J* = 6.7)	16.7		1'", 2'", 4'"
4'"α	1.94 (1H, *m*)	27.3	4'"β	1'", 2'", 5'"
4'"β	1.47 (1H, *m*)		4'"α, 5'"	1'", 2'", 3'", 5'"
5'"	1.01 (3H, *t*, *J* = 7.3)	11.9		2'", 4'"

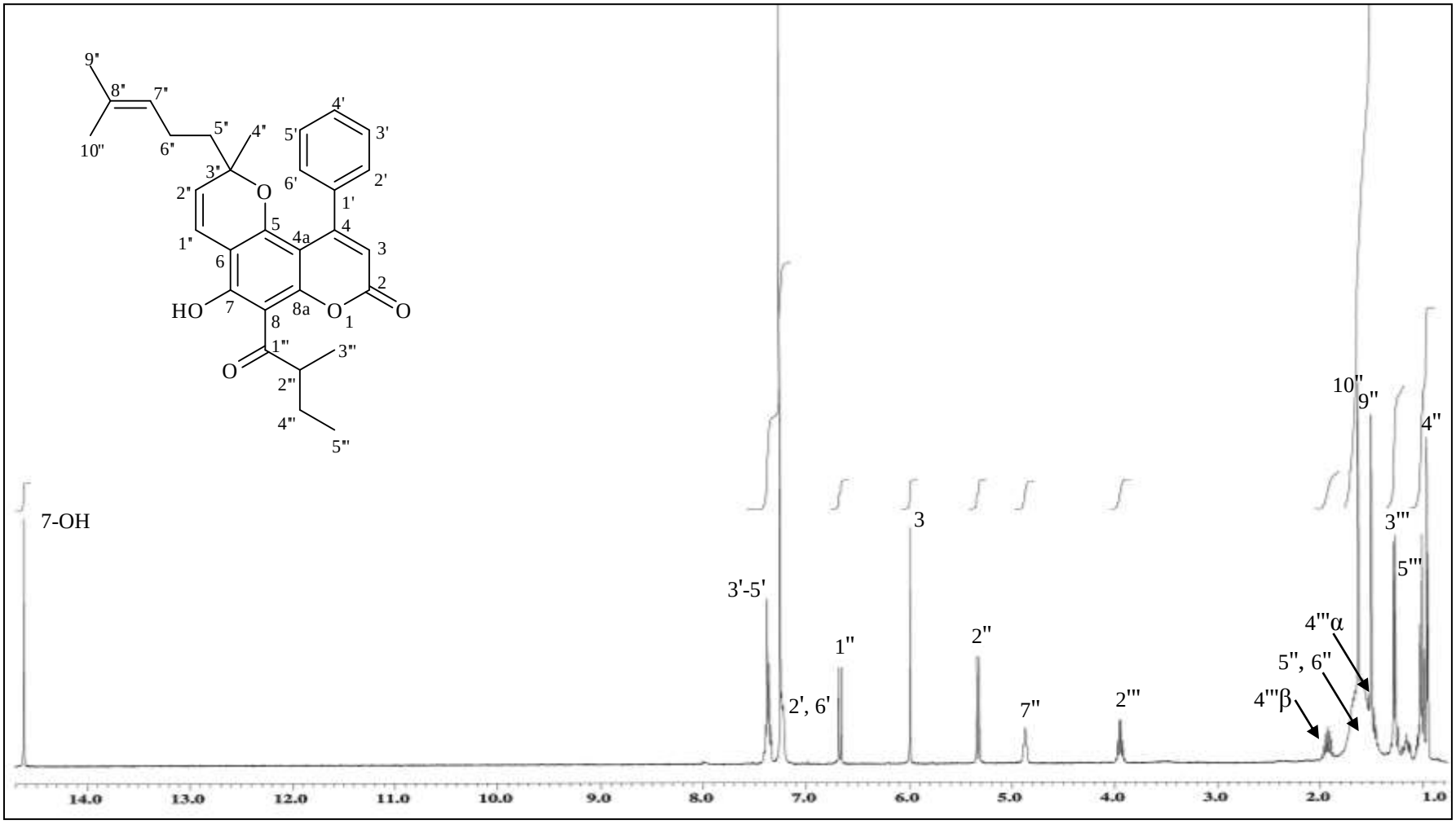


Figure 3.8: ^{1}H NMR Spectrum of Compound E **190**

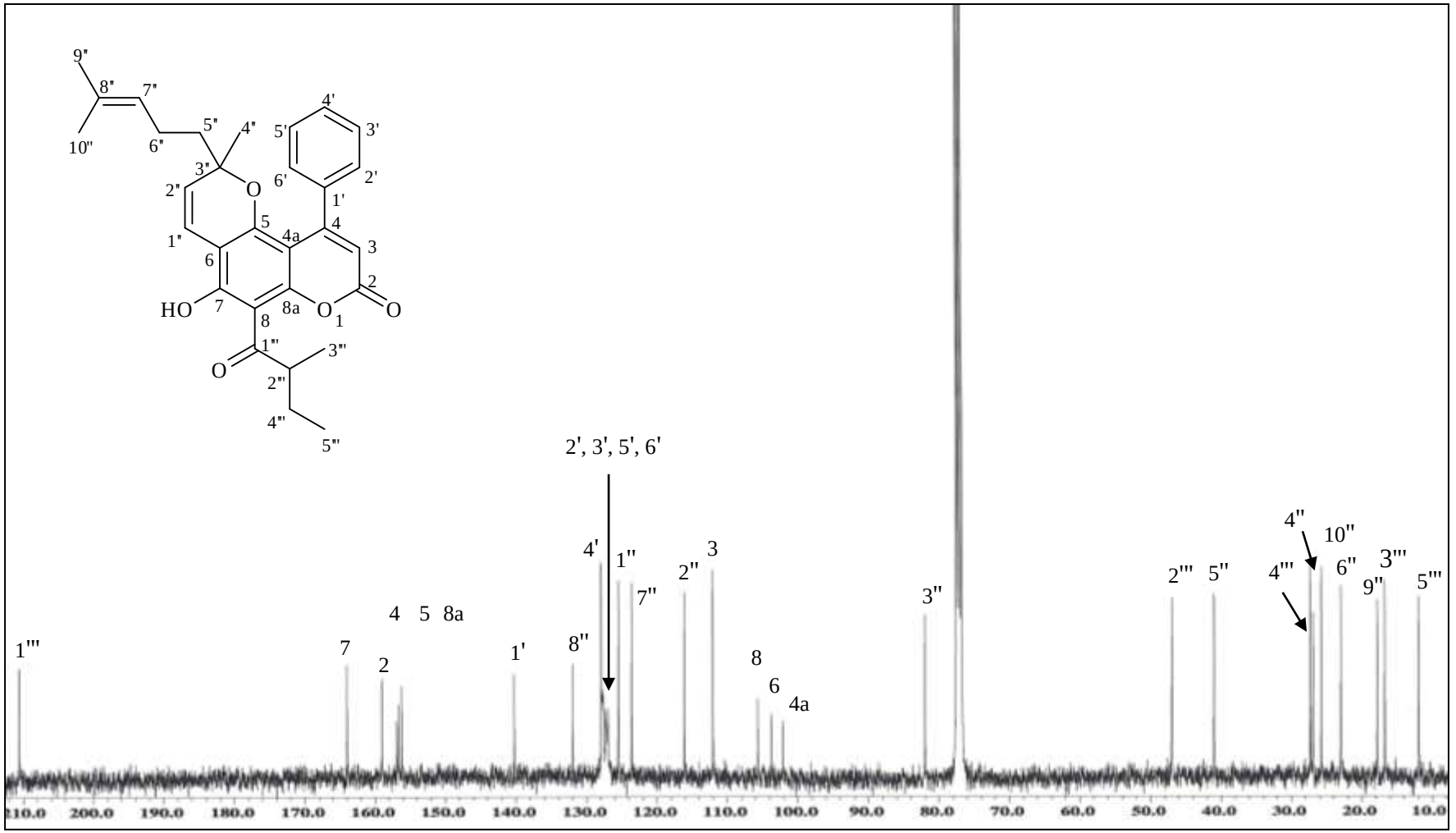
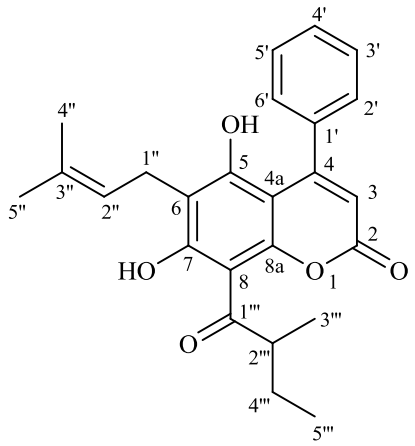


Figure 3.9: ^{13}C NMR Spectrum of Compound E **190**

3.1.6 Compound F: Mammea A/BB 55



55

Compound F was obtained as a colourless oil using column chromatography fractionation, followed by HPLC separation. The HRESIMS spectrum showed a pseudomolecular ion $[M+H]^+$ at *m/z* 407.1855 (calculated 407.1858), which suggested a molecular formula of $C_{25}H_{26}O_5$. The UV spectrum supported an 8-acyl-5,7-dihydroxycoumarin type, with absorptions at λ_{max} 226, 297 and 331 nm[82]. The IR spectrum showed absorptions at υ_{max} 3482 (OH), 1717 (α,β-unsaturated lactone) and 1602 cm^{-1} (chelated acyl group)[47].

The ^{13}C NMR spectrum showed the signals of four methyls, two methylenes, eight methines and eleven quaternary carbons. The presence of a 4-phenylcoumarin type skeleton was indicated by the typical singlet of H-3 proton at δ 5.99, which is vicinal to the lactone functionality of the coumarin skeleton in the ^1H NMR spectrum. In addition, two sets of multiplets corresponding to five protons centred at δ 7.55 (H-3' - H-5') and 7.41 (H-2' and H-6'), respectively, were also observed in the ^1H NMR spectrum of compound F. The ^1H NMR spectrum also exhibited a singlet of a chelated hydroxyl proton at δ 14.57 and an unchelated phenolic hydroxyl singlet at δ 5.94, which were

exchangeable with D_2O. All these observations suggested the presence of an 8-acyl-5,7-dihydroxy-4-phenylcoumarin type[47].

The ^{1}H NMR resonances at δ 3.27 (2H, *d*, *J* = 6.7 Hz, H-1"), 5.07 (1H, br*t*, *J* = 6.7 Hz, H-2"), 1.68 (3H, *s*, H-4") and 1.63 (3H, *s*, H-5"), together with the ^{13}C signals at δ 21.7 (C-1"), 120.9 (C-2"), 134.2 (C-3"), 18.0 (C-4") and 25.8 (C-5") implied the presence of an isoprenyl group. Correlations in the HMBC spectrum between H-1" with C-6 (δ 112.8) and C-7 (δ 167.0) indicated that the isoprenyl group was attached to C-6.

Signals of non-equivalent geminal protons of C-4"' showed two methylene protons as two sets of multiplets at δ 1.51 (1H, *m*, H-4"'α) and 1.93 (1H, *m*, H-4"'β) on the ^{1}H NMR spectrum. In the HMBC spectrum of compound F, these methylene protons correlated with a carbonyl (C-1"', δ 210.6), a methine (C-2"', δ 47.1) and two methyls (C-3"', δ 16.7 and C-5"', δ 11.9). Protons of the two methyls (C-3"' and C-5"') were observed as a doublet at δ 1.27 (*J* = 6.7 Hz) and as a triplet at δ 1.01 (*J* = 7.3 Hz), respectively. These signals confirmed the presence of the 2-methylbutanoyl moiety linked at C-8.

The observed data of compound F indicated that this compound is mammea A/BB **55**, as reported in the literature[93], which has previously been isolated from *Mammea americana* for the first time in 1966.

Table 3.7: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound F **55**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	158.8		
3	5.99 (1H, *s*)	112.2		2, 4a, 1'
4	-	157.1		
4a	-	100.6		
5-OH	5.94 (1H, *s*)	154.2		4, 4a, 6, 1'
6	-	112.8		
7-OH	14.57 (1H, *s*)	167.0		6, 7, 8
8	-	104.3		
8a	-	155.9		
1'	-	136.9		
2'	7.41 (1H, *m*, Ar)	127.6		
3'	7.55 (3H, *m*, Ar)	129.7		1', 2'
4'		130.3		
5'		129.7		
6'	7.41 (1H, *m*, Ar)	127.6		5, 4', 5'
1"	3.27 (2H, *d*, *J* = 6.7)	21.7	2", 4", 5"	4, 6, 7, 2", 3"
2"	5.07 (1H, br*t*, *J* = 6.7)	120.9	1"	1", 4", 5"
3"	-	134.2		
4"	1.68 (3H, *s*)	18.0		2", 3", 5"
5"	1.63 (3H, *s*)	25.8		2", 3", 4"
1'"	-	210.6		
2'"	3.94 (1H, *m*)	47.1	3'"	1'", 3'", 4'", 5'"
3'"	1.27 (3H, *d*, *J* = 6.7)	16.7	2'"	1'", 2'", 4'"
4'"α	1.51 (1H, *m*)	27.3	4'"β, 5'"	1'", 2'", 5'"
4'"β	1.93 (1H, *m*)		4'"α, 5'"	1'", 2'", 3'", 5'"
5'"	1.01 (3H, *t*, *J* = 7.3)	11.9	4'"α, 4'"β	2'", 4'"

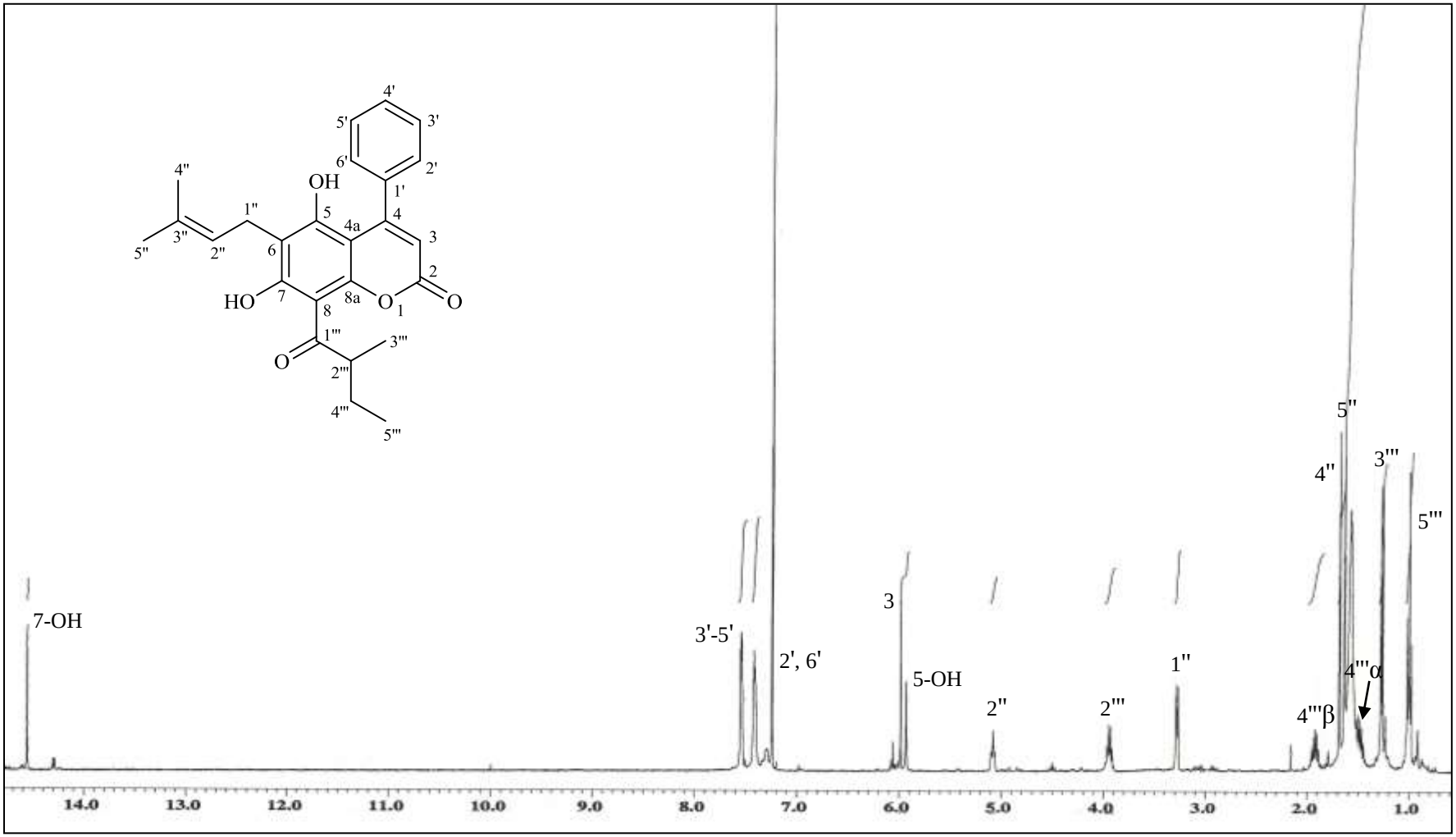
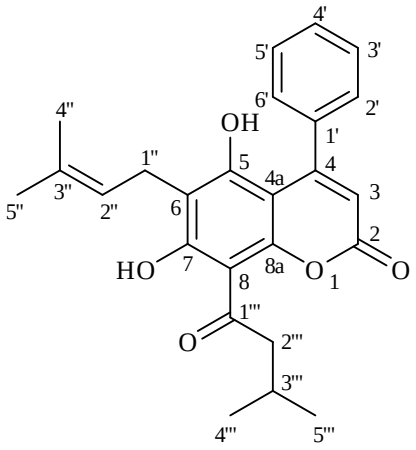


Figure 3.10: ^{1}H NMR Spectrum of Compound F **55**

3.1.7 Compound G: Mammea A/BA 28



28

Compound G was obtained as a colourless oil using column chromatography fractionation followed by HPLC separation. The HRESIMS measurement of its $[M+H]^+$ ion at *m/z* 407.1855 (calculated 407.1858) suggested a molecular formula of $C_{25}H_{26}O_5$. The UV spectrum supported an 8-acyl-5,7-dihydroxycoumarin type, with absorptions at λ_{max} 226, 297 and 331 nm[82]. The IR spectrum showed absorptions at υ_{max} 3482 cm^{-1} (OH), 1717 cm^{-1} (α,β-unsaturated lactone), 1602 cm^{-1} (chelated acyl group) and 1385 cm^{-1} (geminal dimethyl)[47].

The ^1H and ^{13}C NMR spectra of compound G were similar to those of compound F, thus suggesting a close relationship between these two compounds. Indeed, the same substituents for the coumarin skeleton could be characterized in both compounds, namely a typical H-3 singlet of a 4-phenylcoumarin (δ 5.98, *s*, H-3), a monosubstituted phenyl (δ 7.52 and 7.45, 5H, *m*, H-2'-H6'), a prenyl group [δ 3.27 (2H, *d*, *J* = 6.7 Hz, H-1"), 5.07 (1H, br*t*, *J* = 6.7 Hz, H-2"), 1.68 (3H, *s*, H-4") and 1.63 (3H, *s*, H-5")] and a chelated hydroxyl (δ 14.59, *s*, 7-OH).

However, close inspection of ^{1}H NMR spectrum of compound G revealed a difference in the coupling pattern of the substituent group at position C-8; a methylene at δ 3.16 (2H, *d*, *J* = 6.7, H-2'''), 2.30 (1H, *m*, H-3''') and 1.03 (6H, *d*, *J* = 7.3, H-4''' and H-5'''). The observations from the COSY spectrum revealed the presence of an *iso*-butyryl spin system (C-2'''-C-3'''-C-4'''-C-5''') with cross peaks between H-2'''/H-3''' and H-3'''/H-4''', H-5'''. In the HMBC spectrum of compound G, the methylene protons produced cross-peaks with a carbonyl function at δ 206.2 (C-1'''). With this, it was identified that a 3-methylbutanoyl moiety was the substituent of the 4-phenylcoumarin at position C-8.

Comparison of the spectral data of compound G with literature values showed that this compound is mammea A/BA **28** and was first isolated from *Mammea americana* (Clusiaceae) in 1966 by Crombie *et al.*[93].

Table 3.8: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound G **28**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	158.8		
3	5.98 (1H, *s*)	112.3		2, 4a, 1'
4	-	157.2		
4a	-	100.6		
5-OH	5.95 (1H, *s*)	154.2		4, 4a, 6, 1'
6	-	112.7		
7-OH	14.59 (1H, *s*)	166.9		6, 7, 8
8	-	104.7		
8a	-	156.0		
1'	-	136.9		
2'	7.45 (1H, *m*, Ar)	127.6		
3'	7.52 (3H, *m*, Ar)	129.7		1', 2'
4'		130.3		
5'		129.7		
6'	7.45 (1H, *m*, Ar)	127.6		5, 4', 5'
1"	3.27 (2H, *d*, *J* = 6.7)	21.7	2", 4", 5"	4, 6, 7, 2", 3"
2"	5.07 (1H, br*t*, *J* = 6.7)	120.9	1", 4", 5"	1", 4", 5"
3"	-	134.2		
4"	1.68 (3H, *s*)	18.0		2", 3", 5"
5"	1.63 (3H, *s*)	25.9		2", 3", 4"
1'"	-	206.2		
2'"	3.16 (2H, *d*, *J* = 6.7)	53.7	3'"	1'", 3'", 4'", 5'"
3'"	2.30 (1H, *m*)	25.7	2'", 4'", 5'"	2'", 4'", 5'"
4'"	1.03 (6H, *d*, *J* = 7.3)	22.8		2'", 3'", 5'"
5'"		22.8		2'", 3'", 4'"

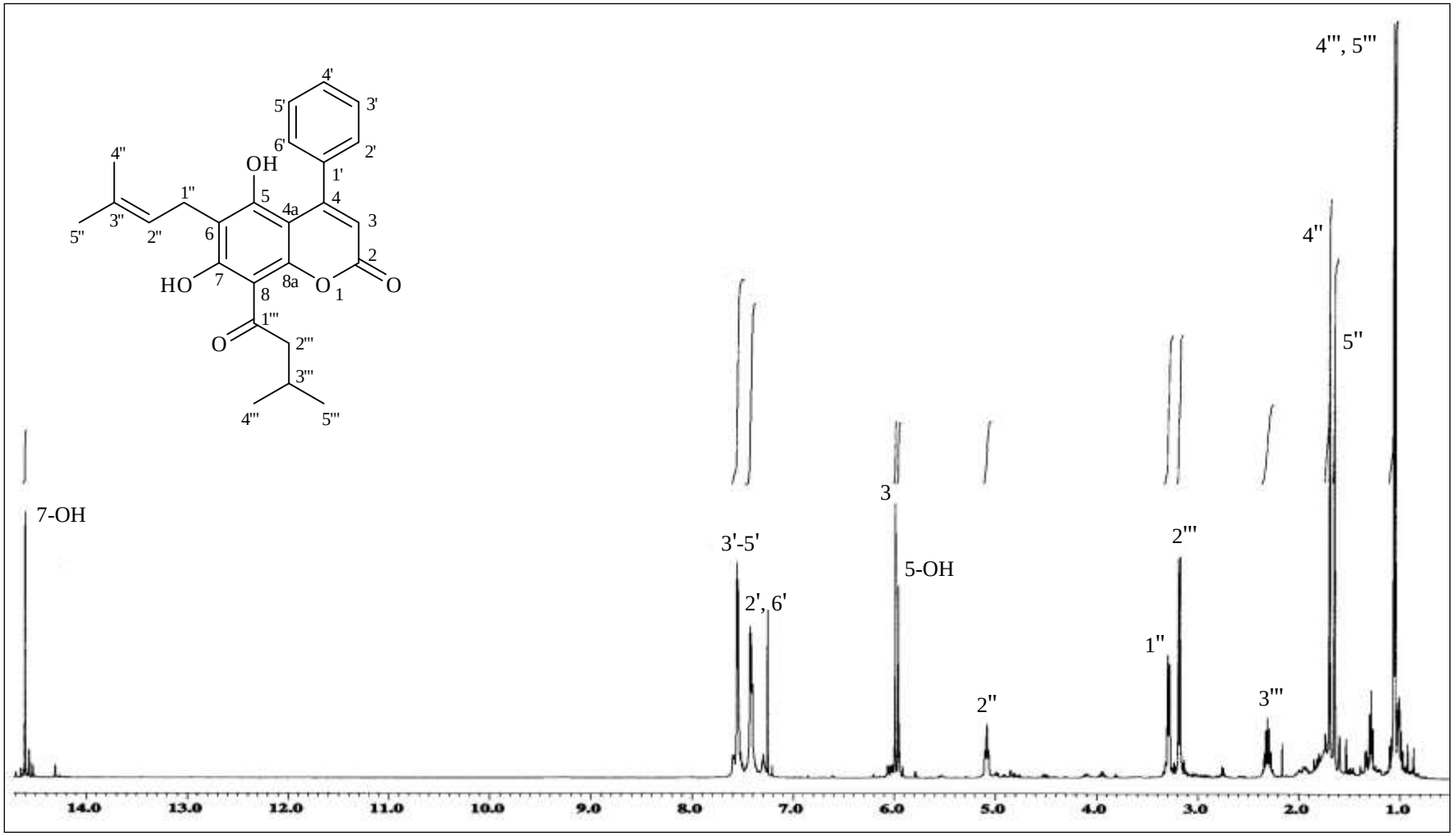


Figure 3.11: ^{1}H NMR Spectrum of Compound G **28**

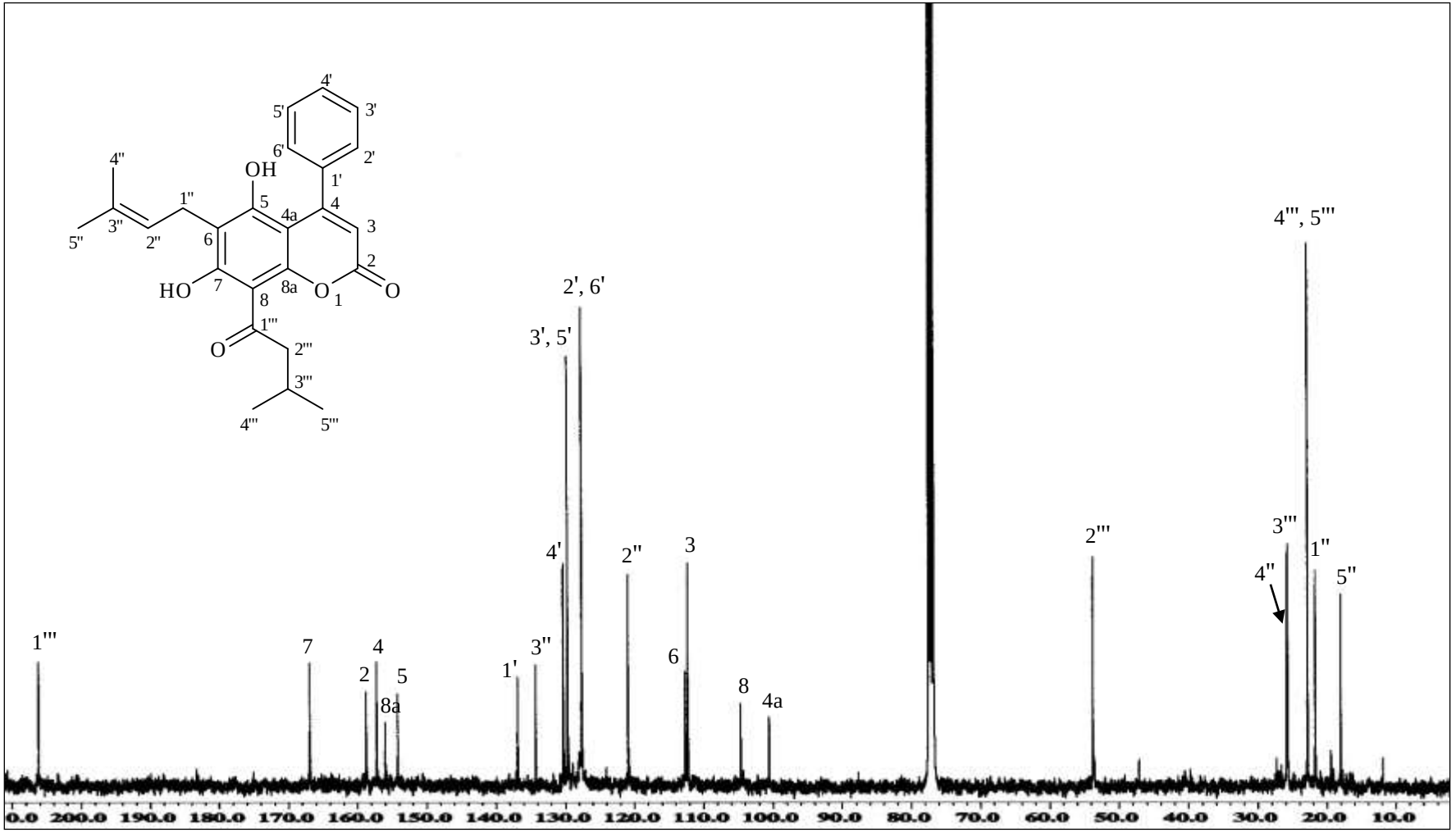
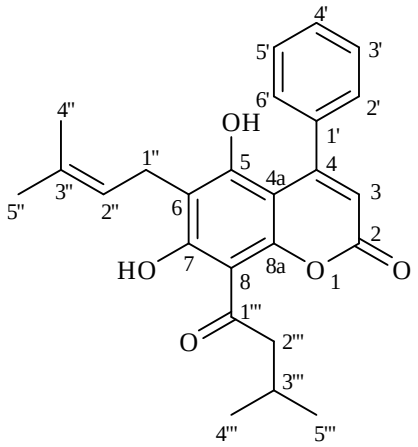


Figure 3.12: ^{13}C NMR Spectrum of Compound G **28**



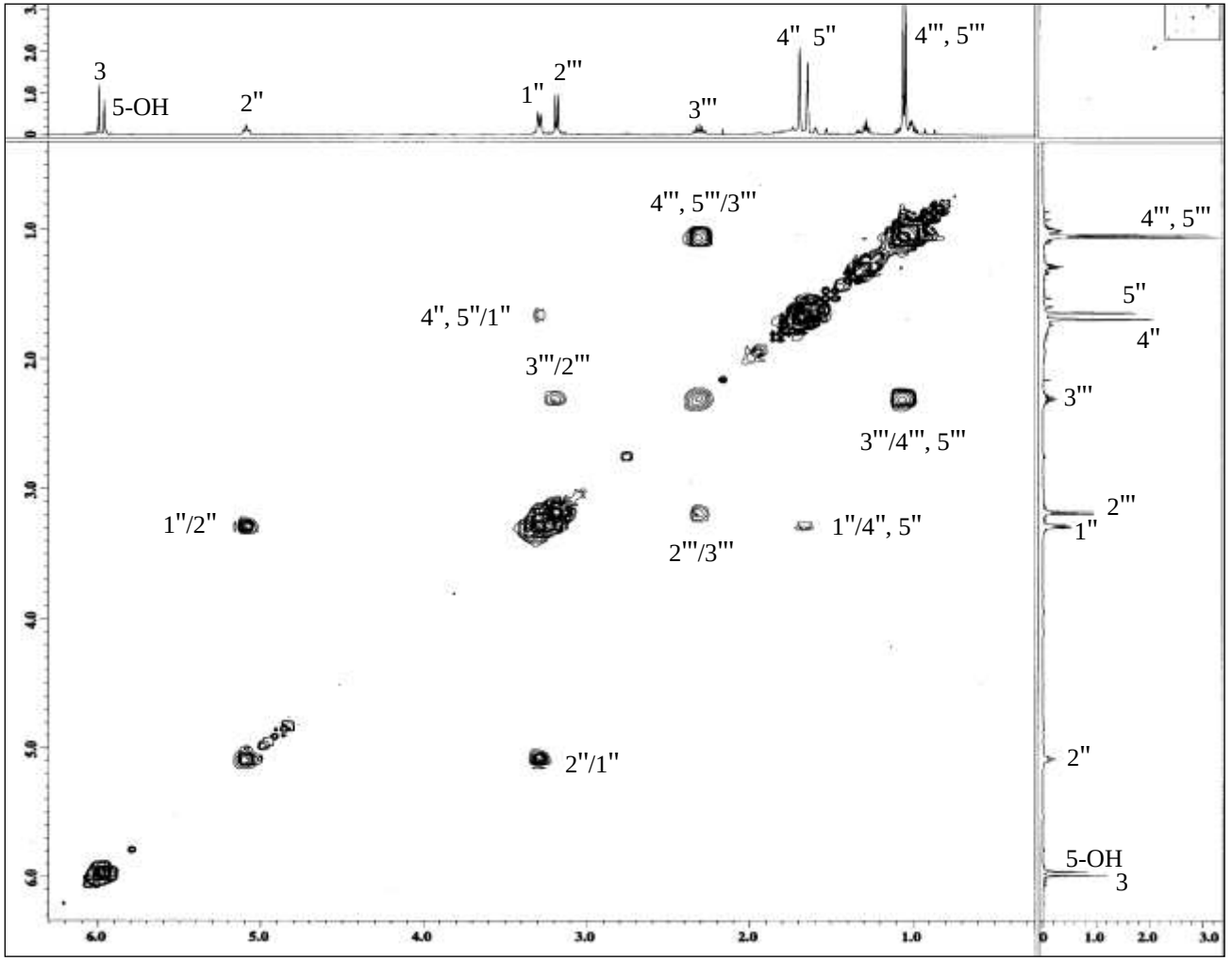
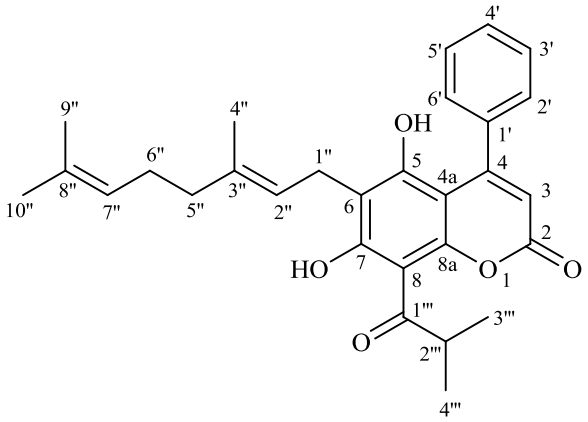


Figure 3.13: COSY Spectrum of Compound G **28**

3.1.8 Compound H: Mesuagenin C 191



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Separation using column chromatography, followed by HPLC, afforded compound H as a white amorphous powder. The HRESIMS measurement revealed an $[M+Na]^+$ ion at m/z 483.2132 (calculated 483.2147), which corresponded to the molecular formula of $C_{29}H_{32}O_5$. The absorptions at λ_{max} 226, 295 and 331 nm in UV spectrum supported an 8-acyl-5,7-dioxycoumarin type[82]. The IR spectrum showed absorptions at υ_{max} 3452 (OH), 1735 (δ-lactone), 1600 (chelated acyl group) and 1386 cm^{-1} (geminal dimethyl)[85, 88]. The IR spectrum showed absorptions at υ_{max} 3470 (OH), 1741 (α-pyrone), 1602 (chelated acyl group) and 1383 cm^{-1} (geminal dimethyl)[47, 88].

The ^1H NMR spectrum of compound H exhibited the typical singlet of H-3 proton at δ 6.00, and two sets of multiplets corresponding to five phenyl protons centred at δ 7.52 (H-3' - H-5') and δ 7.40 (H-2' and H-6'). A singlet of a chelated hydroxyl proton at δ 14.54 and an unchelated phenolic hydroxyl singlet at δ 5.98, which were exchangeable with D_2O, were also apparent in the ^1H NMR spectrum.

The ^{13}C NMR spectrum of compound H revealed twenty-nine carbon signals; five methyls, three methylenes, nine methines and twelve quaternary carbons. In the ^1H

NMR spectrum, three methyl singlets were apparent at δ 1.68 (H-4"), δ 1.59 (H-9") and δ 1.53 (H-10"), along with a doublet integrating for two protons at δ 3.30 (H-1", *J* = 7.3), two methylene protons which appeared as multiplets at δ 1.91 - 2.03 (H-5" and H-6"), and two broad methine triplets at δ 5.08 (H-2", *J* = 6.7) and δ 4.99 (H-7", *J* = 6.7). The linkage of these carbons was apparent from the HMBC spectrum. In the HMBC spectrum, the methyls of C-9" and C-10" showed cross peaks with δ 124.1 (C-7") and δ 131.7 (C-8"), and the methyls of C-4" revealed cross peaks with δ 120.6 (C-2"), δ 128.2 (C-3") and δ 39.8 (C-5"). Moreover, the HMBC spectrum also showed cross correlations between the methylenes of C-5" and C-6" with δ 120.6 (C-2"), δ 128.2 (C-3") and δ 124.1 (C-7"). Also, the methylene protons of C-1" correlated with a methine carbon (C-2", δ 120.6) and two quaternary carbons, C-6 (δ 112.6) and C-7 (δ 167.1). All is evidence supported the presence of a geranyl substituent attached at position C-6 of the coumarin skeleton.

The cross correlations deduced from the COSY spectrum revealed the presence of an *iso*-butyryl spin system with signals at δ 4.11 (1H, *m*, H-2"') and δ 1.28 (6H, *d*, *J* = 6.7, H-3"' and H-4"'). In the HMBC spectrum, the methine proton of C-2"' and the methyl protons of C-3"' and C-4"' gave cross-peaks with a keto function at δ 210.8, belonging to C-1"'. With this, it was identified that an *iso*-butanoyl chain was the other substituent of the 4-phenylcoumarin, and was attached at C-8.

Literature search based on the SciFinder database confirmed that compound H is a new chemical entity, its IUPAC name is 5,7-dihydroxy-8-isobutyryl-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one, which have been named as mesuagenin C[92] **191**.

Table 3.9: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound H **191**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	158.9		
3	6.00 (1H, *s*)	112.2		2, 4a, 1'
4	-	157.3		
4a	-	100.7		
5-OH	5.98 (1H, *s*)	154.4		4, 4a, 6
6	-	112.6		
7-OH	14.54 (1H, *s*)	167.1		6, 7, 8
8	-	103.8		
8a	-	155.8		
1'	-	137.1		
2'	7.40 (1H, *m*, Ar)	127.6		
3'	7.52 (3H, *m*, Ar)	129.6		1', 2'
4'		130.2		
5'		129.6		
6'	7.40 (1H, *m*, Ar)	127.6		4'
1"	3.30 (2H, *d*, *J* = 6.7)	21.7		6, 7, 2"
2"	5.08 (1H, *t*, *J* = 7.4)	120.6	1"	4", 5"
3"	-	138.2		
4"	1.68 (3H, *s*)	16.3		2", 3", 5"
5"	2.03 - 1.91 (4H, *m*)	39.8		2", 3", 6"
6"		26.6		5", 7"
7"	4.99 (1H, *t*, *J* = 7.3)	124.1	6"	
8"	-	131.7		
9"	1.53 (3H, *s*)	17.6		7", 8", 10"
10"	1.59 (3H, *s*)	25.8		7", 8", 9"
1'"	-	210.8		
2'"	4.11 (1H, *m*)	40.5	3'", 4'"	1'", 4'"
3'"	1.28 (6H, *d*, *J* = 7.3)	19.4	2'", 4'"	1'", 2'", 4'"
4'"		19.4	2'", 3'"	3'"

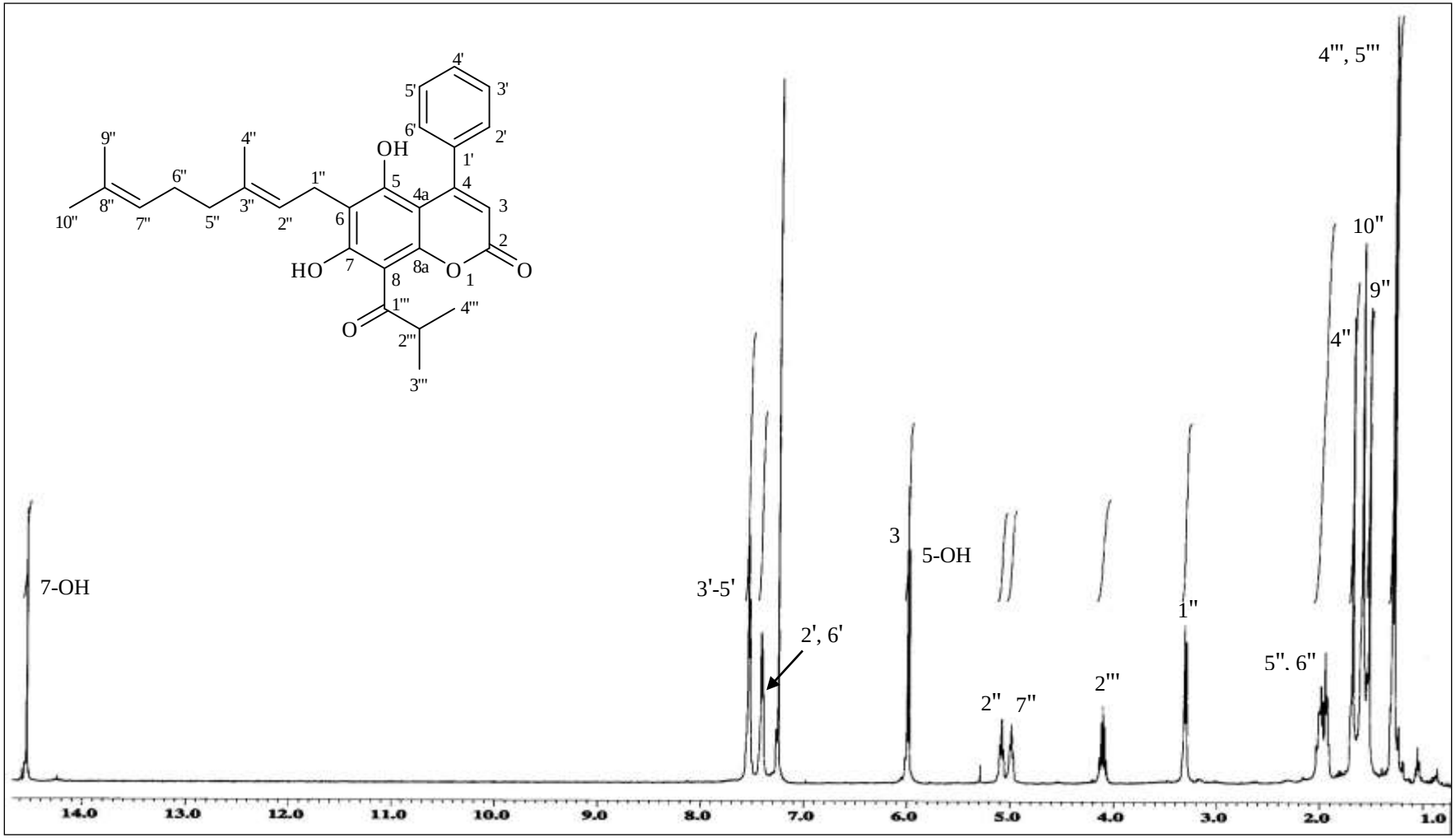


Figure 3.14: ^{1}H NMR Spectrum of Compound H **191**

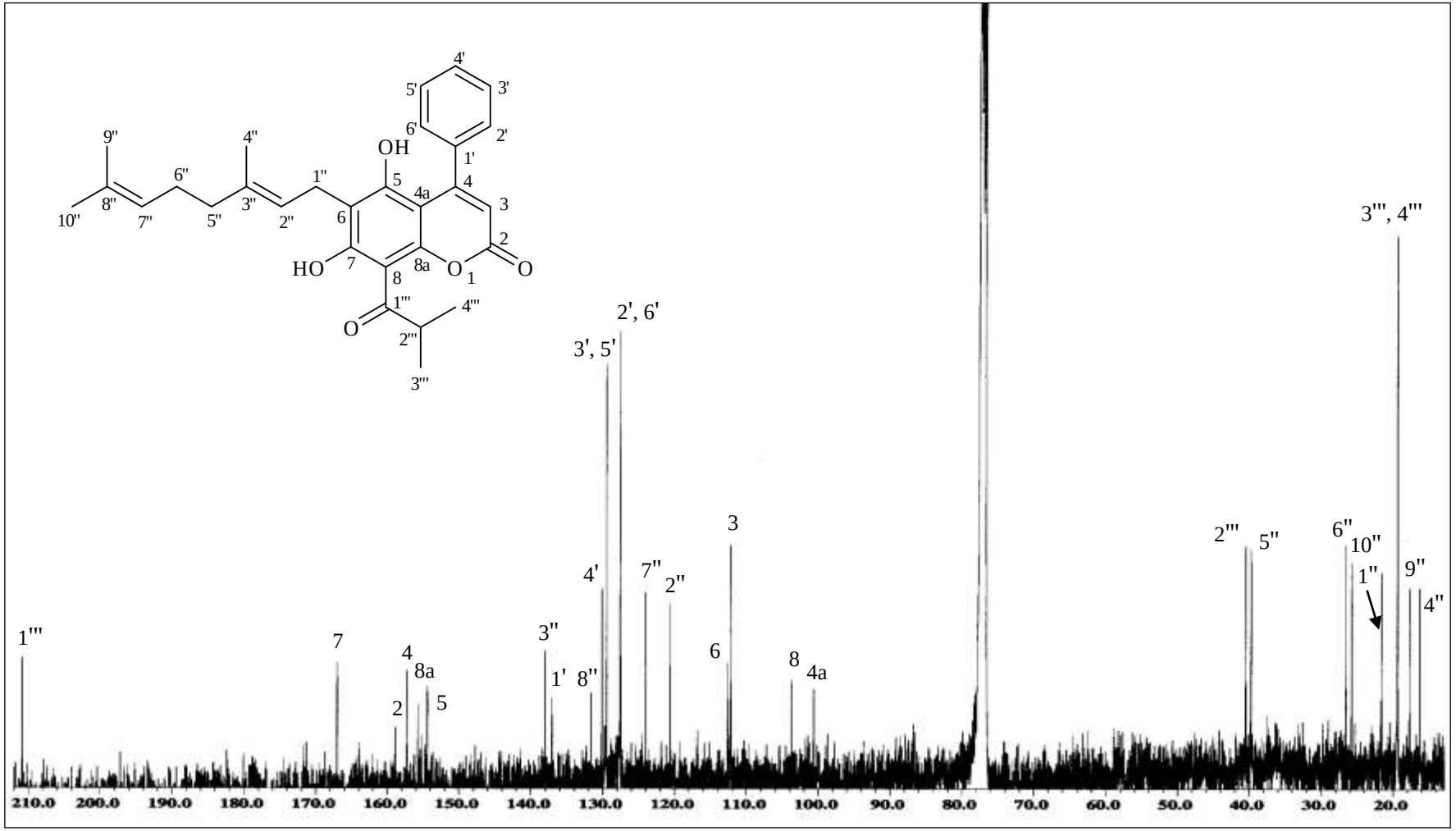
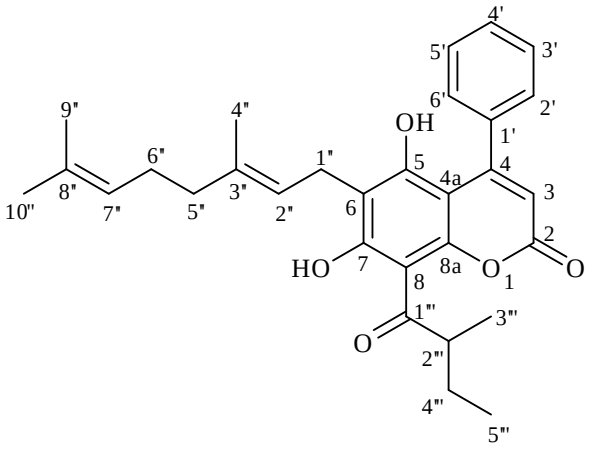


Figure 3.15: ^{13}C NMR Spectrum of Compound H **191**

3.1.9 Compound I: 5,7-Dihydroxy-8-(2-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one 47



47

Compound I was isolated as white crystals with m.p. 90-92°C using column chromatography, followed by HPLC separation. The HRESIMS revealed an $[M+H]^+$ ion at *m/z* 475.2728 (calculated 475.2484), corresponding to the molecular formula of $C_{30}H_{34}O_5$. The UV spectrum (EtOH) showed intense absorption bands at 226, 296 and 333 nm reflecting an 8-acyl-5,7-dihydroxycoumarin type[82]. The IR spectrum showed absorptions at υ_{max} 3471 cm^{-1} (OH), 1741 cm^{-1} (α-pyrone) and 1601 cm^{-1} (chelated acyl group)[47, 88].

^{13}C NMR spectrum of compound I revealed the signals of five methyls, four methylenes, nine methines and twelve quaternary carbons (for a total of thirty carbons). In the ^{1}H NMR spectrum, a typical H-3 singlet was observed at δ 6.00, characteristic of the C-3 proton of a 4-phenyl substituted coumarin. Moreover, two sets of multiplets corresponding to five protons assignable to H-3' - H-5', along with the H-2' and H-6' protons, were observed at δ 7.52 and δ 7.40, respectively. In addition, a proton singlet characteristic of a chelated hydroxyl at δ 14.59 (7-OH), and another hydroxyl singlet at

δ 5.98 (5-OH) was also observed. All these observations suggested the presence of an 8-acyl-5,7-dihydroxy-4-phenylcoumarin type[47].

In the ^{1}H NMR spectrum of compound I, three methyl singlets were apparent at δ 1.69 (H-4"), δ 1.61 (H-9") and δ 1.53 (H-10"), along with two broad triplets at δ 5.08 (1H, *J* = 6.7) and δ 4.99 (1H, *J* = 6.7), corresponding to the olefinic protons H-2" and H-7", respectively, in the HMBC spectrum. A doublet, integrating for two protons, was detected at δ 3.30 (H-1", *J* = 7.3) in the ^{1}H NMR spectrum. In the HMBC spectrum, these methylene protons correlated with a methine carbon (C-2", δ 120.6) and two quaternary carbons, C-6 (δ 112.6) and C-7 (δ 167.0), suggesting a geranyl substituent attached at position C-6.

Signals of two methylene protons as two sets of multiplets at δ 1.47 (1H, *m*, H-4'"α) and 1.91 (1H, *m*, H-4'"β) were apparent in the ^{1}H NMR spectrum. In the HMBC spectrum, these protons correlated with a carbonyl (C-1'", δ 210.7), a methine (C-2'", δ 47.1) and two methyls (C-3'", δ 16.72 and C-5'", δ 11.9). Protons of the two methyls (C-3'" and C-5'") were observed as a doublet at δ 1.27 (*J* = 6.7) and as a triplet at δ 1.01 (*J* = 7.3), respectively. These elements confirmed the presence of the 2-methylbutanoyl moiety linked at C-8.

By comparison of the observed data with the literature values, it was confirmed that compound I was 5,7-dihydroxy-8-(2-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one **47**, (DMDP-1), which was previously isolated by Verotta *et al*. from *Mesua ferrea* in 2004[46]. Compound I **47** was isolated as a mixture with compound J **48**, in a crystal form, and was published in Acta Crystallographica Section E (Appendix).

Table 3.10: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound I **47**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	158.8		
3	6.00 (1H, *s*)	112.3		2, 4a, 1'
4	-	157.3		
4a	-	100.6		
5-OH	5.98 (1H, *s*,)	154.4		6
6	-	112.6		
7-OH	14.59 (1H, *s*)	167.0		6, 7, 8
8	-	104.2		
8a	-	155.8		
1'	-	137.1		
2'	7.40 (1H, *m*, Ar)	127.6		4'
3'	7.52 (3H, *m*, Ar)	129.2		1'
4'		130.1		
5'		129.2		
6'	7.40 (1H, m, Ar)	127.6		
1"	3.30 (2H, *d*, *J* = 7.3)	21.6		4, 6, 7, 2", 3"
2"	5.08 (1H, brt, *J* = 6.7)	120.6	1"	1", 4", 5"
3"	-	138.1		
4"	1.69 (3H, *s*)	16.3		2", 3", 5"
5"	2.03 - 1.93 (4H, *m*)	39.8		2", 3", 7"
6"		26.6		3", 5", 7", 8"
7"	4.99 (1H, br*t*, *J* = 6.7)	124.0		10"
8"	-	131.7		
9"	1.61 (3H, *s*)	17.7		7", 8", 10"
10"	1.53 (3H, *s*)	25.8		7", 8", 9"
1'"	-	210.7		
2'"	3.94 (1H, *m*)	47.1	3'"	1'", 3'", 4'", 5'"
3'"	1.27 (3H, *d*, *J* = 6.7)	16.7		1'", 2'", 4'"
4'"α	1.47 (1H, *m*)	27.3	5'"	1'", 2'", 3'", 5'"
4'"β	1.91 (1H, *m*)		4'"α, 5'"	1'", 3'", 5'"
5'"	1.01 (3H, *t*, *J* = 7.3)	11.9		2'", 4'"

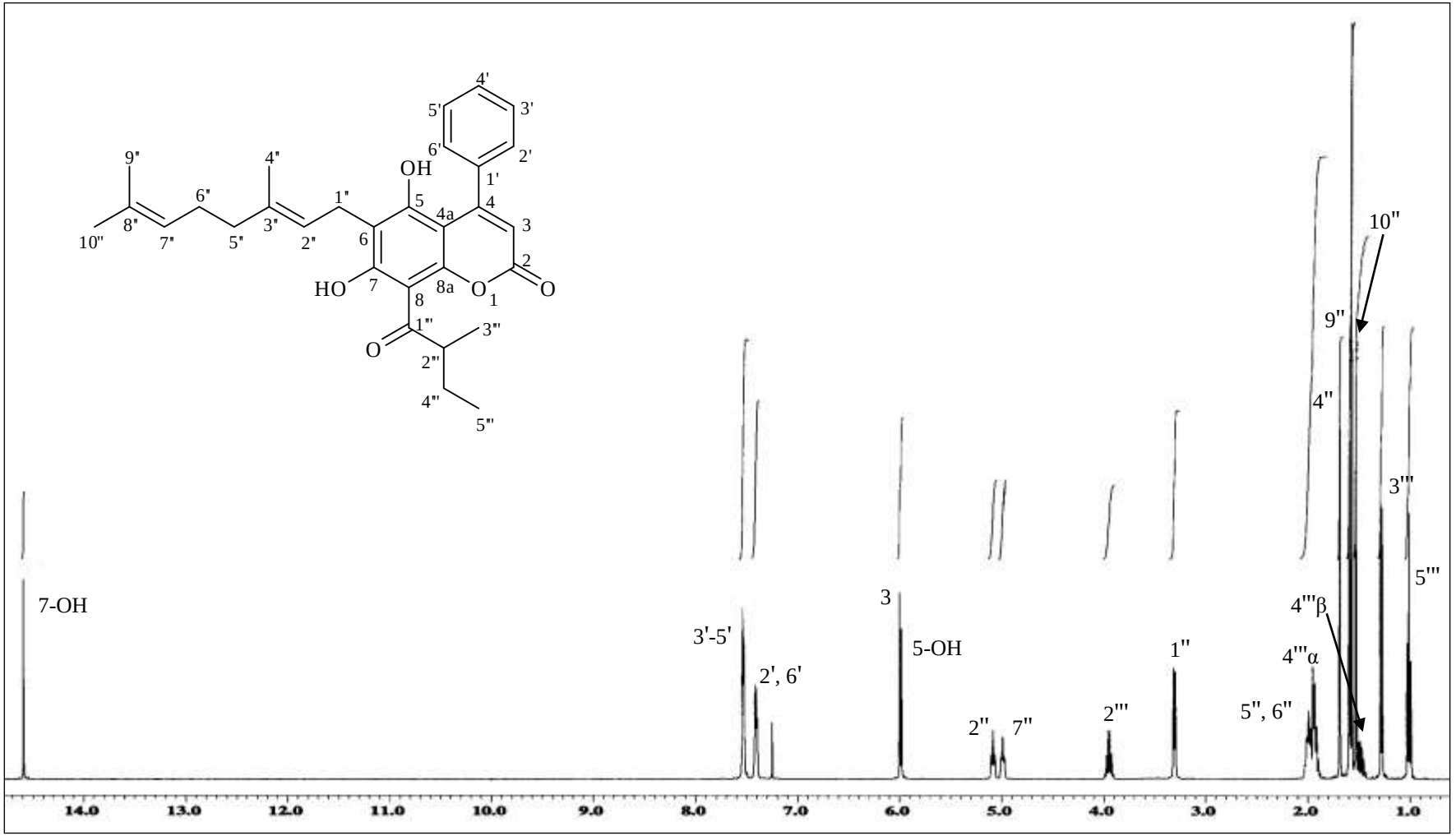


Figure 3.16: ^{1}H NMR Spectrum of Compound I **47**

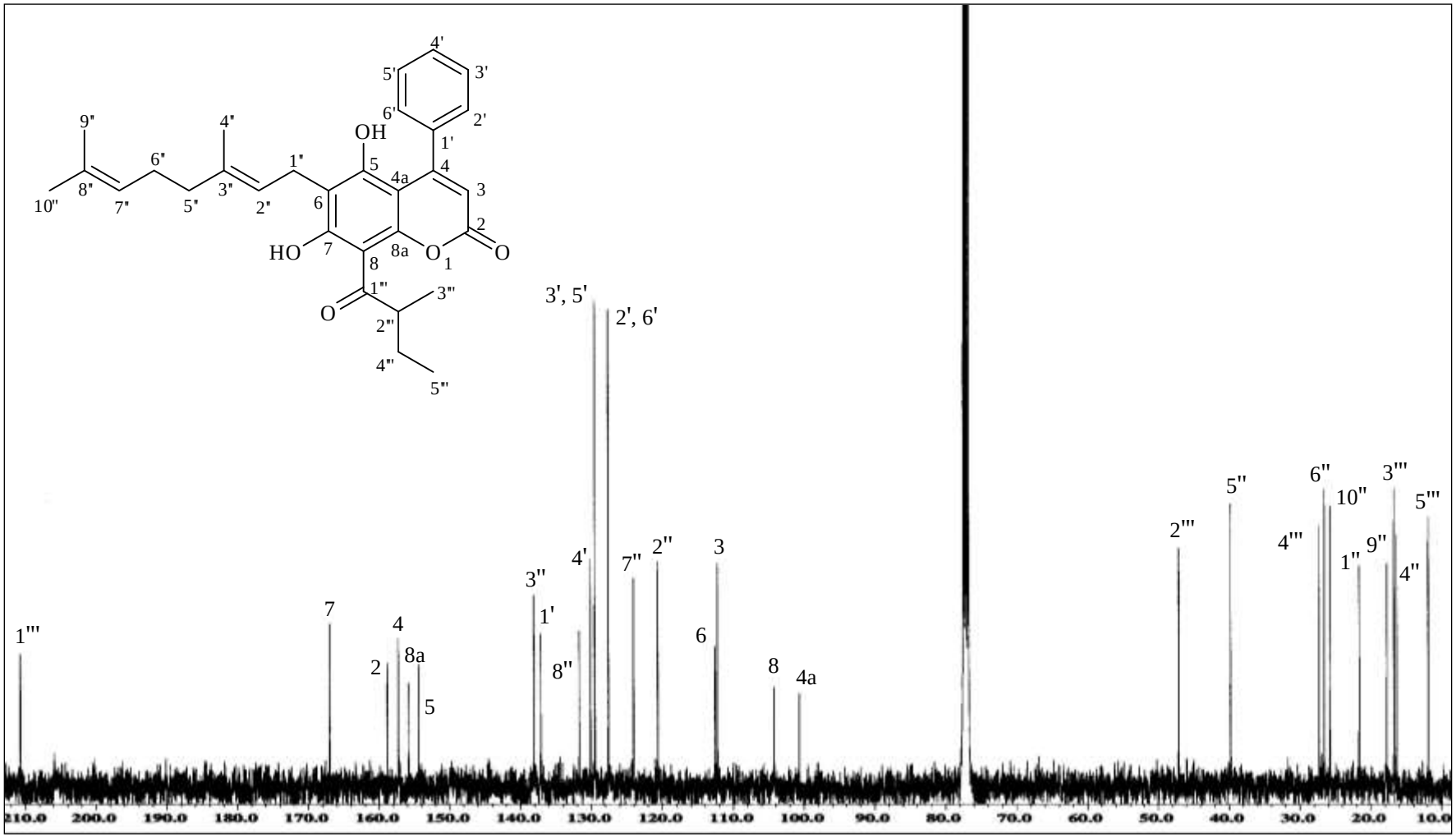


Figure 3.17: ^{13}C NMR Spectrum of Compound I **47**

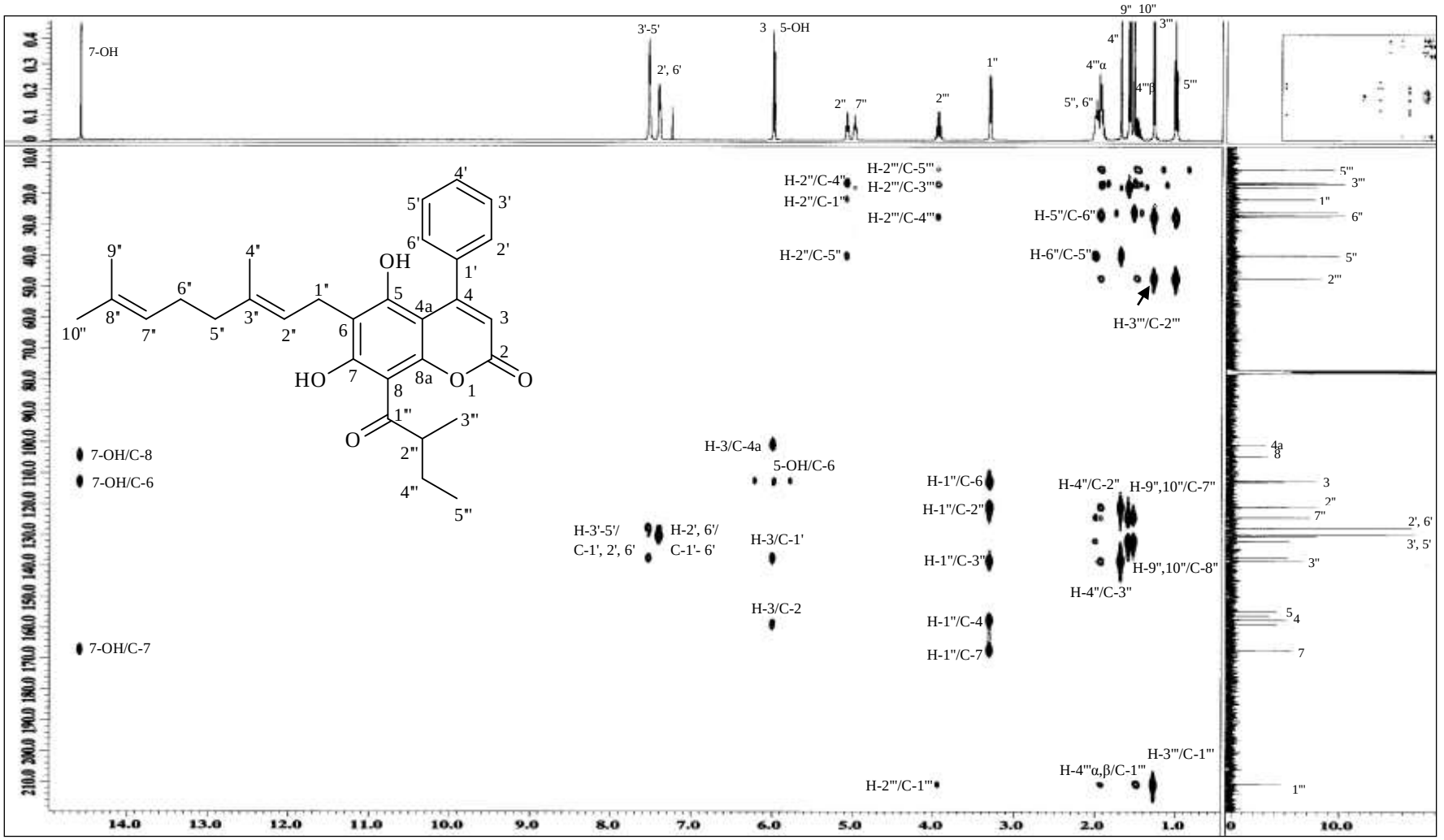
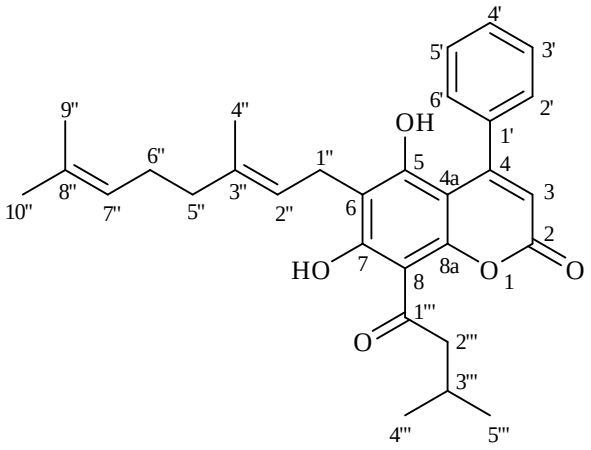


Figure 3.18: HMBC NMR Spectrum of Compound I **47**

3.1.10 Compound J: 5,7-Dihydroxy-8-(3-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one 48



48

Compound J was separated as yellowish oil. The molecular formula of compound J was determined by the HRESIMS measurement of its $[M+H]^+$ ion at *m/z* 475.2783 (calculated 475.2484), as $C_{30}H_{34}O_5$. The IR spectrum exhibited a strong peak at 3472 cm^{-1}, due to hydroxyl group stretching. Peaks were also observed at 1603 and 1742 cm^{-1}, indicating a chelated acyl group and an α-pyrone, respectively[47]. The UV spectrum (EtOH) supported an 8-acyl-5,7-dioxycoumarin moeity, with absorptions at λ_{max} 272 and 313 nm[82].

The gross features of the ^1H NMR and ^{13}C NMR spectra of compounds I and J confirmed a close structural relationship between these compounds. Indeed, the same substituents for the coumarin could be characterized in both compounds through COSY, HMQC and HMBC experiments, namely a monosubstituted phenyl, a chelated hydroxyl, an unchelated hydroxyl and a geranyl chain.

However, compound I and J exhibited noticeable differences in the coupling patterns of protons of the acyl chain attached to C-8. The doublet and triplet signals of C-3''' and C-

5''' methyls were absent in ^{1}H NMR spectrum of compound J. Instead, a doublet integrating for six protons was present, suggesting a 3-methylbutanoyl moiety, instead of a 2-methylbutanoyl moiety, as in compound I.

Extensive analysis of the spectroscopic data established the complete assignments of all the ^{1}H and ^{13}C signals of compound J, which eventually led to the identification of the compound as 5,7-dihydroxy-8-(3-methylbutanoyl)-6-[(E)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one (DMDP-2) **32**, which was isolated from *Mesua ferrea* in 2004 by Verotta *et al*.[46]. Compound I **47** was isolated as a mixture with compound J **48**, in a crystal form, and was published in Acta Crystallographica Section E (Appendix).

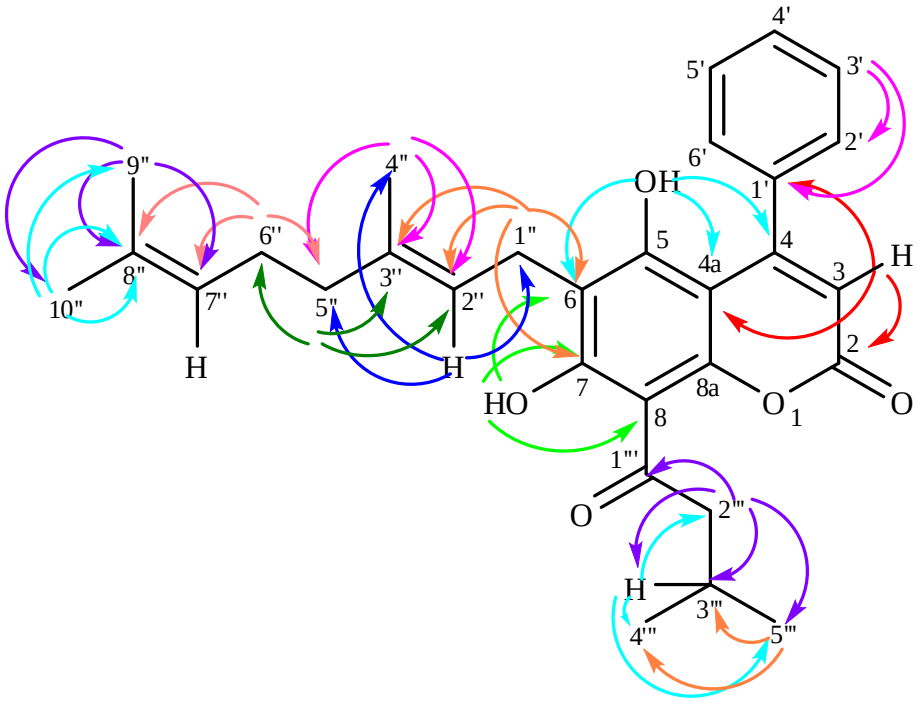


Figure 3.19: The HMBC Correlations of Compound J **48**

Table 3.11: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound J **48**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2		158.8		
3	5.99 (1H, *s*)	112.2		2, 4a, 1'
4		157.4		
4a		100.6		
5-OH	5.98 (1H, *s*)	154.3		4, 4a, 6
6		112.5		
7-OH	14.62 (1H, *s*)	166.9		6, 7, 8
8		104.6		
8a		156.0		
1'		137.1		
2'	7.40 (1H, *m*, Ar)	127.6		4'
3'	7.52 (3H, *m*, Ar)	129.5		2', 1'
4'		130.1		
5'		129.5		
6'	7.40 (1H, *m*, Ar)	127.6		
1"	3.30 (2H, *d*, *J* = 6.7)	21.6		4, 6, 7, 2", 3"
2"	5.08 (1H, br*t*, *J* = 7.3)	120.7	1"	1", 4", 5"
3"		138.1		
4"	1.69 (3H, *s*)	16.3		2", 3", 5"
5"	2.03 - 1.92 (4H, *m*)	39.8		2", 3", 4", 6"
6"		26.6		5", 7", 8"
7"	4.99 (1H, br*t*, *J* = 7.3)	124.1		
8"		131.7		
9"	1.59 (3H, *s*)	17.7		7", 8", 10"
10"	1.53 (3H, *s*)	25.8		7", 8", 9"
1'"		206.2		
2'"	3.18 (2H, *d*, *J* = 6.7)	53.8	3'"	1'", 3'", 4'", 5'"
3'"	2.31 (1H, *m*)	25.7	4'", 5'"	2'", 4'", 5'"
4'"	1.04 (6H, *d*, *J* = 6.7)	22.9	3'", 5'"	3'", 5'"
5'"		22.9	3'", 4'"	3'", 4'"

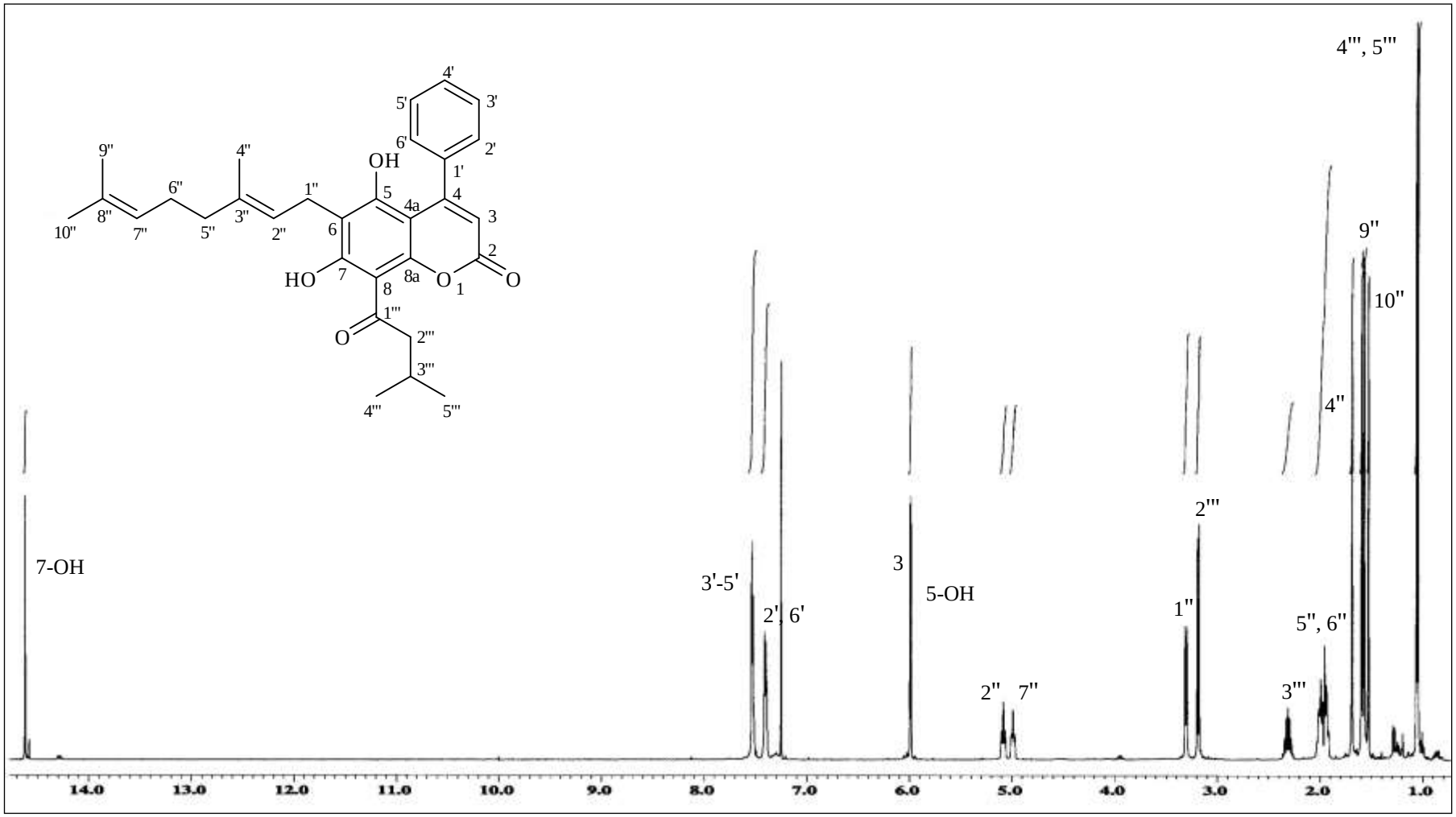
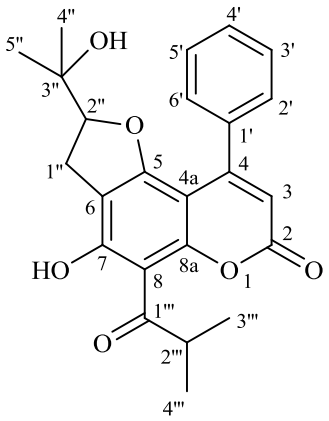


Figure 3.20: ^{1}H NMR Spectrum of Compound J **48**

3.1.11 Compound K: Ochrocarpin E 192



192

Separation using column chromatography, followed by HPLC afforded compound K as a white amorphous powder. The HRESIMS measurement revealed an $[M+Na]^+$ ion at *m/z* 431.1486 (calculated 431.1471), which corresponded to the molecular formula of $C_{24}H_{24}O_6$. The absorptions at λ_{max} 227, 298 and 331 nm in UV spectrum supported an 8-acyl-5,7-dioxycoumarin type[82]. The IR spectrum showed absorptions at υ_{max} 3452 (OH), 1735 (δ-lactone), 1600 (chelated acyl group) and 1386 cm^{-1} (geminal dimethyl)[85, 88].

The ^{13}C NMR spectrum of compound K showed twenty-four carbon signals; four methyls, one methylene, eight methines and eleven quaternary carbons. A typical H-3 singlet was observed at δ 6.06 in the ^1H spectrum, characteristic of the C-3 proton of a 4-substituted coumarin. The presence of a mono-substituted phenyl group at C-4 was corroborated by the presence of two sets of multiplets, centred at δ 7.43 (H-3' - H-5') and 7.29 (H-2' and H-6'), corresponding to three and two aromatic protons, respectively. Furthermore, the ^1H NMR spectrum supported the presence of a chelated hydroxyl by revealing a proton singlet characteristic of a chelated hydroxyl at δ 14.25 (7-OH), and the broad OH absorption depicted in the IR spectrum further supported this finding.

These observations on the ^{1}H NMR spectrum, together with the UV data, suggested the presence of an 8-acyl-5,7-dioxy-4-phenylcoumarin type skeleton[47].

The presence of two doublets of doublets at δ 2.94 (1H, *J* = 9.8, 15.9 Hz, H-1"α) and 3.09 (1H, *J* = 9.8, 15.9 Hz, H-1"β) respectively, and a triplet at δ 4.51 (1H, *J* = 9.8 Hz, H-2"), indicated the presence of a dihydrofuran moiety. The HMBC resonances of δ 1.00 (Me-4") and 0.92 (Me-5") showed cross peaks with δ 92.8 (C-2") and δ 71.7 (C-3"), which indicated the connection of Me-4" and Me-5" with C-3". In addition, the HMBC correlations showed that the dihydrofuran ring was located between C-5 (δ 161.9) and C-6 (δ 110.3) by exhibiting the following correlation, H-1"α,β/C-5, C-6, C-2" and C-3".

The cross correlations deduced from COSY spectrum revealed the presence of *iso*-butyryl spin system with signals at δ 4.11 (1H, *m*, H-2"') and δ 1.28 (6H, *d*, *J* = 6.72 Hz, H-3"' and H-4"'). In the HMBC spectrum, the methine proton and the methyl protons gave cross-peaks with a keto function at δ 210.8, belonging to C-1"'. With this, it was identified that an *iso*-butanoyl chain was the other substituent of the 4-phenylcoumarin, and was attached at C-8.

Based on the analysis of all the spectral data and comparison with the literature values, compound K has been identified as ochrocarpin E **192**, which was previously isolated from *Ochrocarpos punctatus* by Chaturvedula *et al*. in 2002[94].

Table 3.12: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound K **192**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	159.3		
3	6.06 (1H, *s*)	111.0		2, 4a, 1'
4	-	155.1		
4a	-	99.0		
5	-	161.9		
6	-	110.3		
7-OH	14.25 (1H, *s*)	164.0		6, 7, 8
8	-	104.2		
8a	-	157.2		
1'	-	138.2		
2'	7.29 (1H, *m*, Ar)	127.5		3'
3'	7.43 (3H, *m*, Ar)	129.0		1', 4'
4'		128.0		
5'		129.0		
6'	7.29 (1H, *m*, Ar)	127.5		
1"α	2.94 (1H, *dd*, *J* = 9.8, 15.9)	27.0		5, 6, 2", 3"
1"β	3.09 (1H, *dd*, *J* = 9.8, 15.9)			5, 6, 3"
2"	4.51 (1H, *t*, *J* = 9.8)	92.8	1"α, 1"β	
3"-OH	-	71.7		
4"	1.00 (3H, *s*)	23.3		2", 3", 5"
5"	0.92 (3H, *s*)	24.9		2", 3", 4"
1"'	-	210.8		
2"'	4.11 (1H, *m*)	40.3	3"', 4"'	1"', 3"', 4"'
3"'	1.28 (6H, *d*, *J* = 6.7)	19.4		1"', 2"', 4"'
4"'		19.4		1"', 2"', 3"'

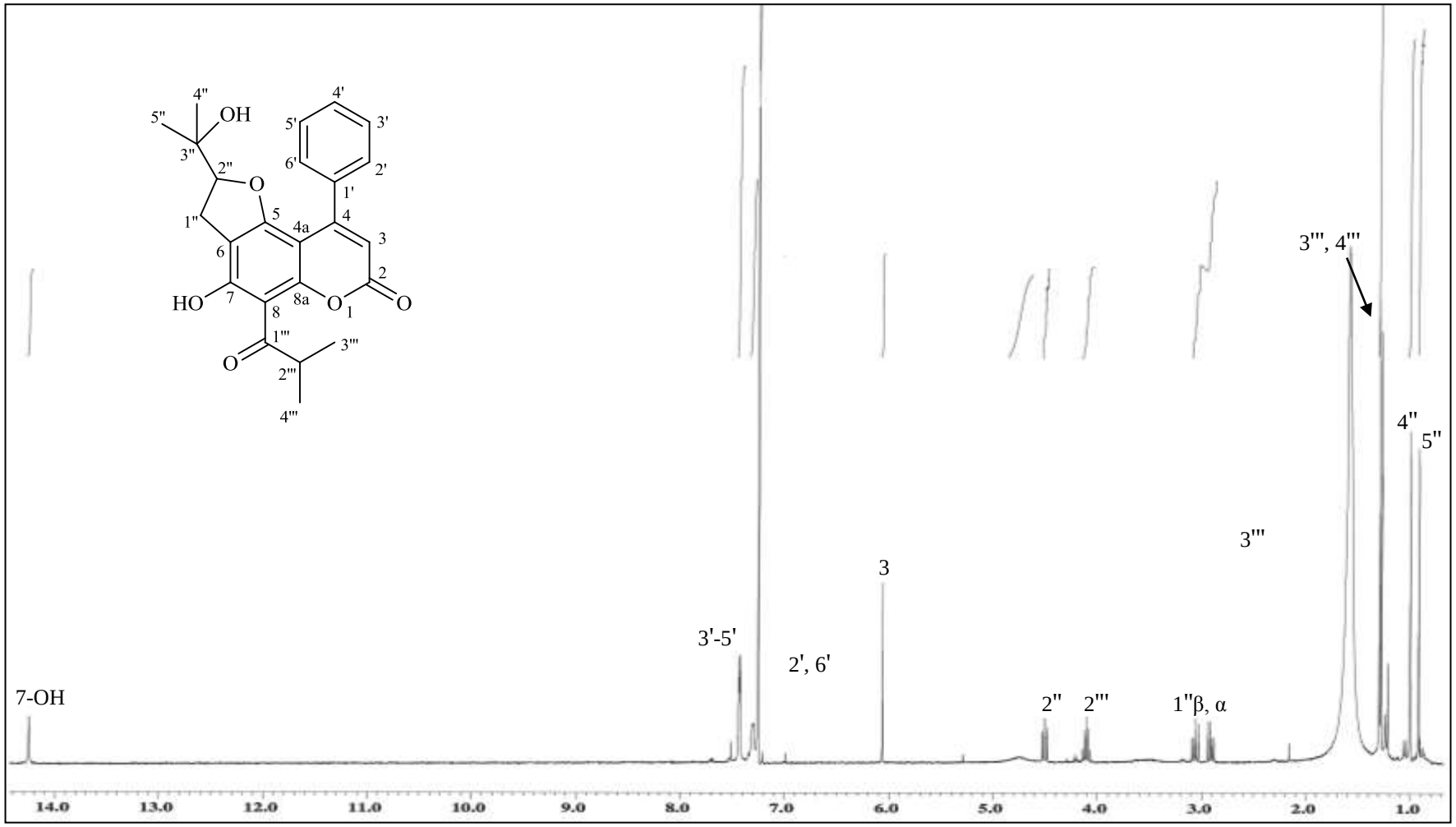


Figure 3.21: ^{1}H NMR Spectrum of Compound K **192**

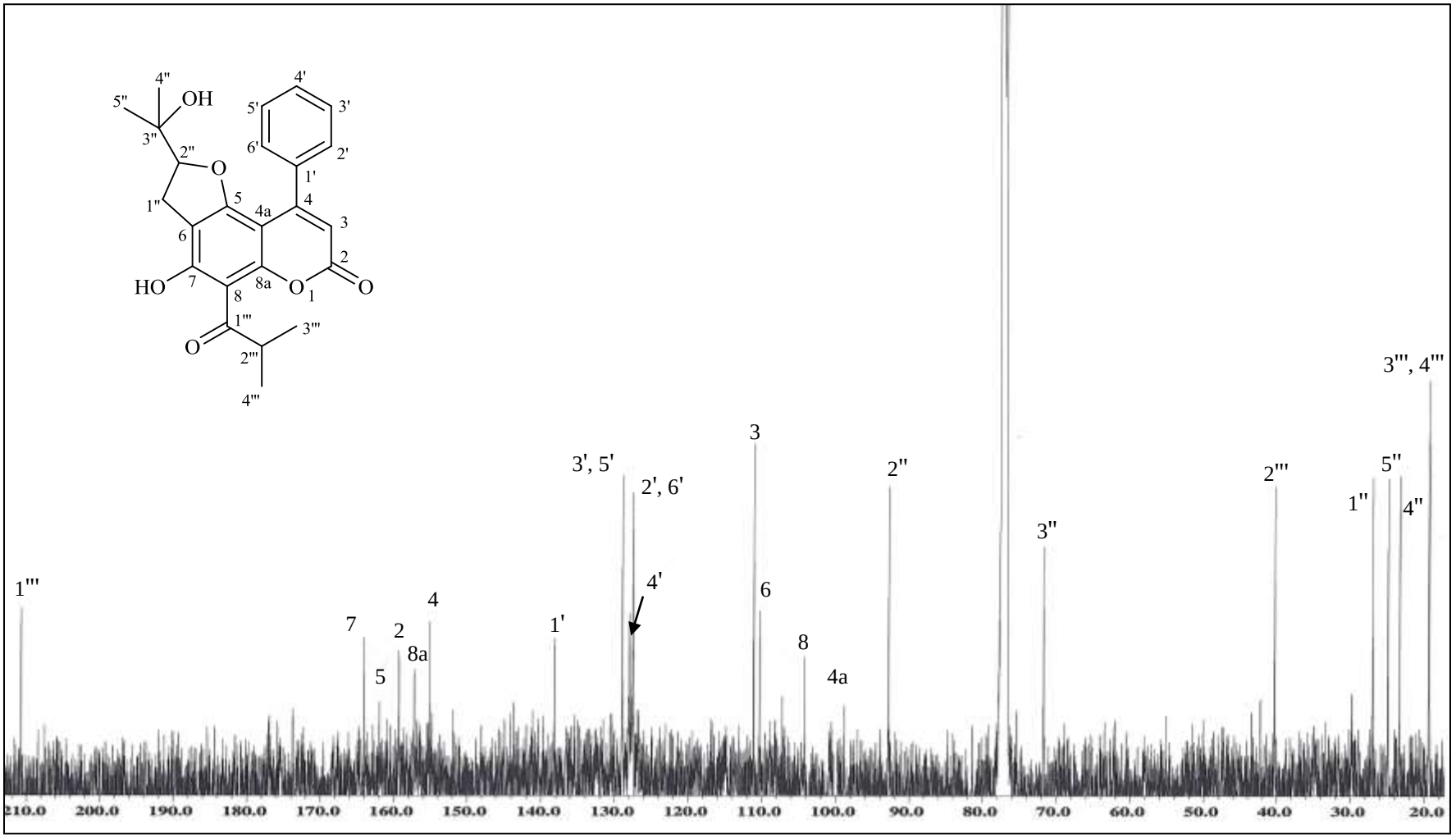
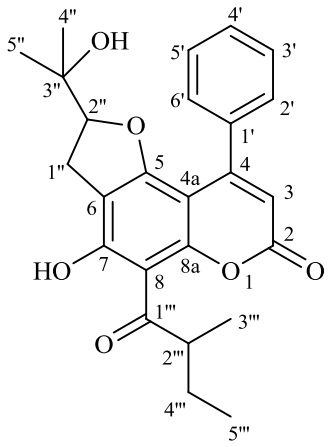


Figure 3.22: ^{13}C NMR Spectrum of Compound K **192**

3.1.12 Compound L: Mammea A/BB cyclo F 193



193

Compound L was separated as a white amorphous solid. The molecular formula of compound L was determined by the HRESIMS measurement of its $[M+Na]^+$ ion at *m/z* 445.1664 (calculated 445.1627), as $C_{25}H_{26}O_6$. The IR spectrum showed absorptions at υ_{max} 3453 (OH), 1732 (δ-lactone) and 1603 cm^{-1} (chelated acyl group)[85, 88]. The absorptions at λ_{max} 225, 298 and 330 nm in the UV spectrum supported an 8-acyl-5,7-dioxycoumarin type[85].

The ^{13}C NMR spectrum showed the signals of four methyls, two methylenes, eight methines and eleven quaternary carbons. The presence of a 4-phenylcoumarin type skeleton was indicated by the singlet of a typical H-3 proton which was vicinal to the lactone functionality of the coumarin skeleton, and was observed at δ 6.06 in the ^1H NMR spectrum of compound L. In addition, two sets of multiplets corresponding to five protons, centred at δ 7.43 (H-3' - H-5') and δ 7.31 (H-2' and H-6'), were also observed in the ^1H NMR spectrum. The ^1H NMR spectrum also exhibited a singlet of a chelated hydroxyl proton at δ 14.29, which was exchangeable with D_2O. All these observations suggested the presence of an 8-acyl-5,7-dioxy-4-phenylcoumarin type[47].

The ^{1}H NMR spectrum also showed the presence of a triplet at δ 4.50 (1H, *J* = 9.8 Hz, H-2") and two doublets of doublets at δ 3.09 (1H, *J* = 9.8, 15.9 Hz, H-1"β) and 2.49 (1H, *J* = 9.8, 15.9 Hz, H-1"α) respectively, thus indicating the presence of a dihydrofuran moiety. The HMBC resonances at δ 1.00 (3H, *s*, H-4") and 0.92 (3H, *m*, H-5") showed cross peaks with δ 92.8 (C-2") and δ 71.7 (C-3"), which indicated the connection of Me-4" and Me-5" with C-3". In addition, the HMBC correlations established that the dihydrofuran ring was located between C-5 (δ 161.9) and C-6 (δ 110.2) by exhibiting the following correlation, H-1"α,β/C-5, C-6, C-2" and C-3".

Signals of non-equivalent geminal protons of C-4"' showed two methylene protons as two sets of multiplets at δ 1.49 (1H, *m*, H-4"'α) and 1.92 (1H, *m*, H-4"'β) in the ^{1}H NMR spectrum. In the HMBC spectrum, these methylene protons correlated with a carbonyl (C-1"', δ 210.6), a methine (C-2"', δ 46.9) and two methyl group (C-3"', δ 16.7 and C-5"', δ 11.9). Protons of the two methyl group (C-3"' and C-5"') were observed as a doublet at δ 1.27 (*J* = 6.8 Hz) and as a triplet at δ 1.01 (*J* = 7.4 Hz), respectively. These signals confirmed the presence of the 2-methylbutanoyl moiety linked at C-8.

The observed data of compound L indicated that this compound is mammea A/BB cyclo F **193**, which was first isolated from *Calophyllum dispar* in 2011 by Guilet *et al*.[85].

Table 3.13: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound L **193**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	159.2		
3	6.06 (1H, *s*)	111.1		2, 4a, 1'
4	-	155.1		
4a	-	98.7		
5	-	161.9		
6	-	110.2		
7-OH	14.29 (1H, *s*)	164.0		6, 7, 8, 1'''
8	-	104.7		
8a	-	157.2		
1'	-	138.2		
2'	7.31 (1H, *m*, Ar)	127.5		3'
3'	7.43 (3H, *m*, Ar)	128.9		1', 4'
4'		128.0		
5'		128.9		
6'	7.31 (1H, *m*, Ar)	127.5		
1"α	2.49 (1H, *dd*, *J* = 9.8, 15.9)	27.0		5, 6, 2", 3"
1"β	3.09 (1H, *dd*, *J* = 9.8, 15.9)			5, 6, 3"
2"	4.50 (1H, *t*, *J* = 9.8)	92.8	1"α, 1"β	5, 4"
3"-OH	-	71.7		
4"	1.00 (3H, *s*)	23.3		2", 3", 5"
5"	0.92 (3H, *s*)	24.9		2", 3", 4"
1'''	-	210.6		
2'''	3.96 (1H, *m*)	46.9	3'''	1''', 3''', 4''', 5'''
3'''	1.27 (3H, *d*, *J* = 6.8)	16.7		1''', 2''', 4'''
4'''α	1.49 (1H, *m*)	27.3	4'''β, 5'''	1''', 2''', 3''', 5'''
4'''β	1.92 (1H, *m*)		4'''α, 5'''	1''', 2''', 3''', 5'''
5'''	1.01 (3H, *t*, *J* = 7.4)	11.9		2''', 4'''

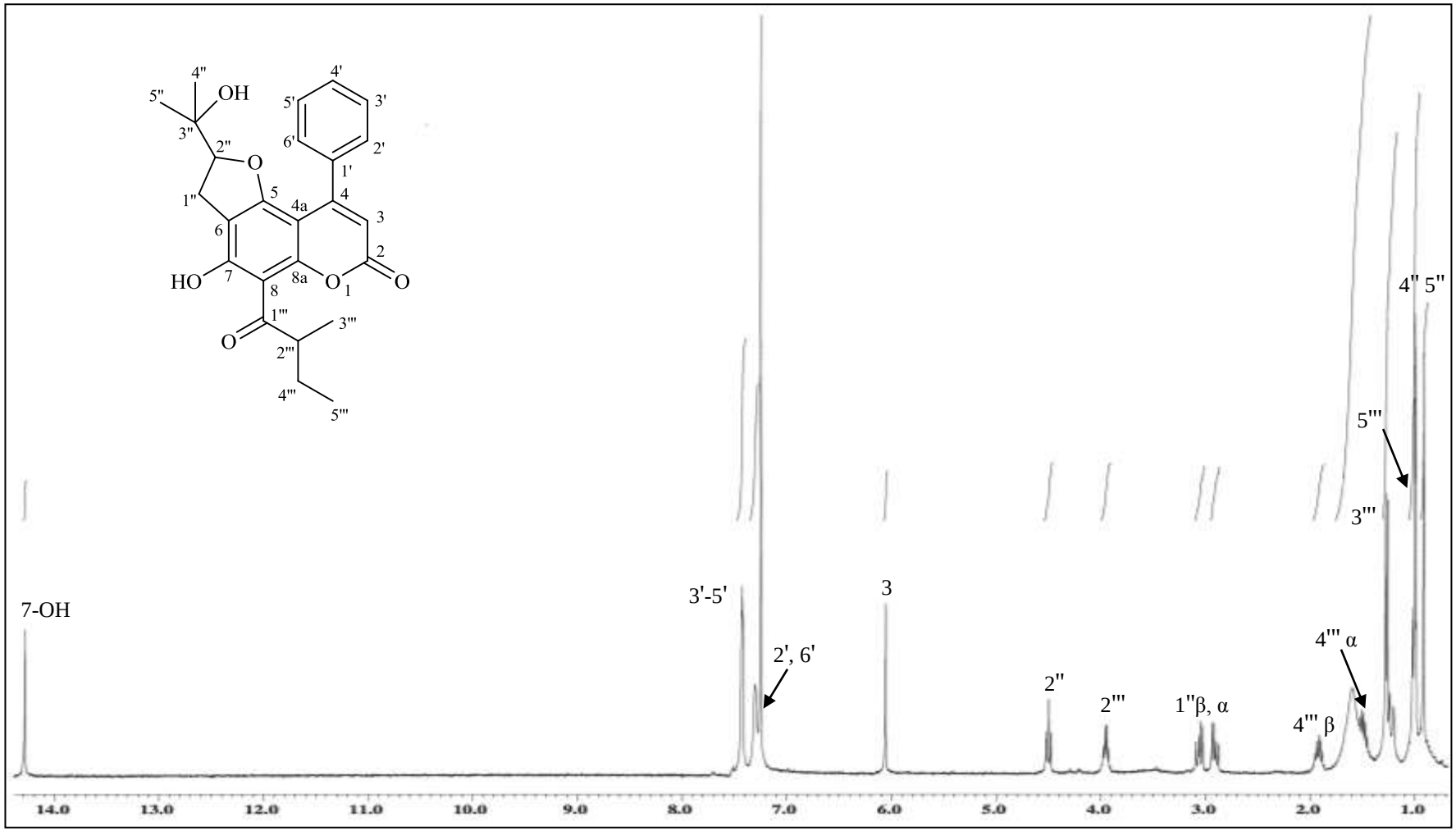


Figure 3.23: ^{1}H NMR Spectrum of Compound L **193**

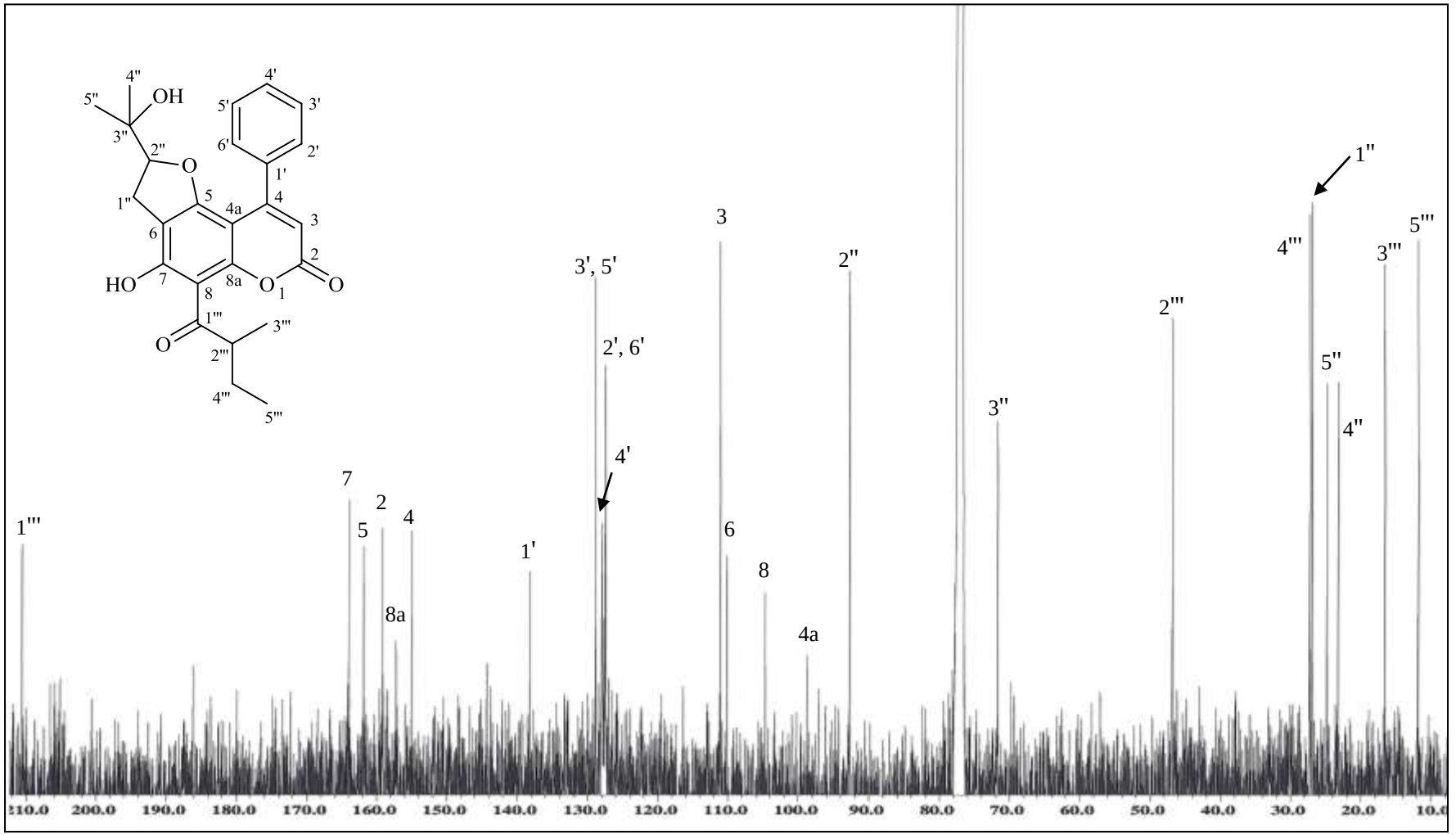
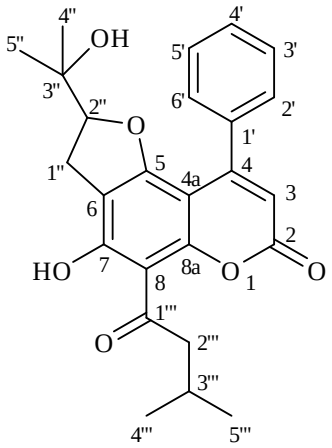


Figure 3.24: ^{13}C NMR Spectrum of Compound L **193**

3.1.13 Compound M: Mammea A/BA cyclo F 194



194

Compound M was obtained as a white amorphous powder and the HRESIMS measurement revealed an $[M+Na]^+$ ion at *m/z* 445.1660 (calculated 445.1627), which corresponded to the molecular formula of $C_{25}H_{26}O_6$. The UV absorptions (EtOH) at λ_{max} 227, 300 and 331 nm indicated that compound M possess an 8-acyl-5,7-dioxycoumarin skeleton[82]. The IR spectrum showed absorptions at υ_{max} 3444 (OH), 1744 (δ-lactone), 1602 (chelated acyl group) and 1384 cm^{-1} (geminal dimethyl)[47].

A total of twenty-five carbons were observed in the ^{13}C NMR spectrum of compound M; four methyls, two methylenes, eight methines and eleven quaternary carbons. The ^1H NMR spectrum of compound M was reminiscent with those of compound L, thus suggesting a similarity between these compounds. The same substituents were characterized in both compounds, namely a typical H-3 signal of a 4-phenylcoumarin (δ 6.05, *s*, H-3), a monosubstituted phenyl (δ 7.43 and 7.30, 5H, *m*, H-2'-H6'), a cyclized furan group [δ 3.09 (1H, *dd*, *J* = 9.8, 15.9 Hz, H-1"β), 2.49 (1H, *dd*, *J* = 9.8, 15.9 Hz, H-1"α), 4.49 (1H, *t*, *J* = 9.8 Hz, H-2"), 0.99 (3H, *s*, H-4") and 0.91 (3H, *s*, H-5")] and a chelated hydroxyl group (δ 14.29, *s*, 7-OH).

The ^{1}H and ^{13}C NMR spectra of M were reminiscent of those of compound L, in which compound M possessed a monosubstituted phenyl, a cyclized furan and a chelated hydroxyl group. These observations suggested a close skeletal resemblance between compounds L and M.

However, the ^{1}H NMR spectrum showed obvious differences in the proton resonances of the remaining substituent; a methylene at δ 3.16 (2H, *d*, *J* = 6.7 Hz, H-2'''), δ 2.30 (1H, *m*, H-3''') and δ 1.05 (6H, *d*, *J* = 7.3 Hz, H-4''' and H-5'''). The observation from COSY spectrum revealed the presence of an *iso*-butyryl spin system (C-2'''-C-3'''-C-4'''-C-5''') with cross peaks between H-2'''/H-3''', H-3'''/H-4''' and H-5'''. In the HMBC spectrum of compound M, the methylene protons caused cross-peaks with a keto function at δ 206.2 (C-1'''). With this, it was identified that a 3-methylbutanoyl chain was the substituent of the coumarin nucleus at C-8.

Based on the analysis of all the spectral data and comparison with the literature values, compound M has been identified as mammea A/BA cyclo F **194**, which was previously purified from *Calophyllum dispar* in 2011[85].

Table 3.14: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound M **194**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	159.1		
3	6.05 (1H, *s*)	111.1		2, 4a, 1'
4	-	155.0		
4a	-	98.7		
5	-	162.0		
6	-	110.1		
7-OH	14.29 (1H, *s*)	163.8		6, 7, 8
8	-	105.1		
8a	-	157.5		
1'	-	138.2		
2'	7.30 (1H, *m*, Ar)	127.5		3'
3'	7.43 (3H, *m*, Ar)	128.9		1', 4'
4'		128.0		
5'		128.9		
6'	7.30 (1H, *m*, Ar)	127.5		
1"α	2.49 (1H, *dd*, *J* = 9.8, 15.9)	26.9		5, 6, 2", 3"
1"β	3.09 (1H, *dd*, *J* = 9.8, 15.9)			5, 6, 3"
2"	4.49 (1H, *t*, *J* = 9.8)	92.8	1"α, 1"β, 4", 5"	
3"-OH	-	71.7		
4"	0.99 (3H, *s*)	23.3		2", 3", 5"
5"	0.91 (3H, *s*)	24.9		2", 3", 4"
1"'	-	206.2		
2"'	3.16 (2H, *d*, *J* = 6.7)	53.5	3"', 4"', 5"'	1"', 3"', 4"', 5"'
3"'	2.30 (1H, *m*)	25.7	4"', 5"'	4"', 5"'
4"'	1.05 (6H, *d*, *J* = 6.7)	22.8		2"', 3"', 5"'
5"'		22.8		4"'

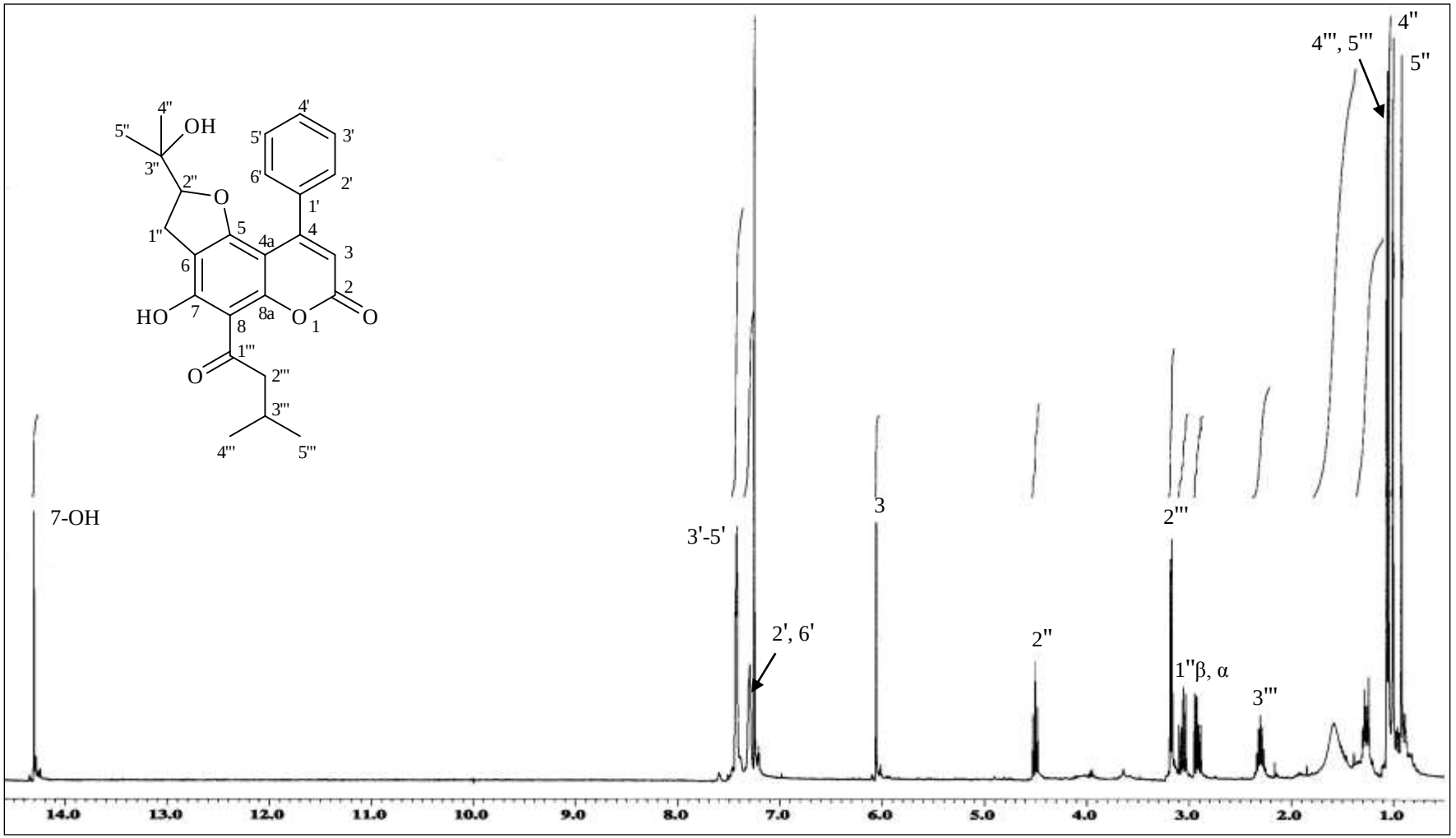


Figure 3.25: ^{1}H NMR Spectrum of Compound M **194**

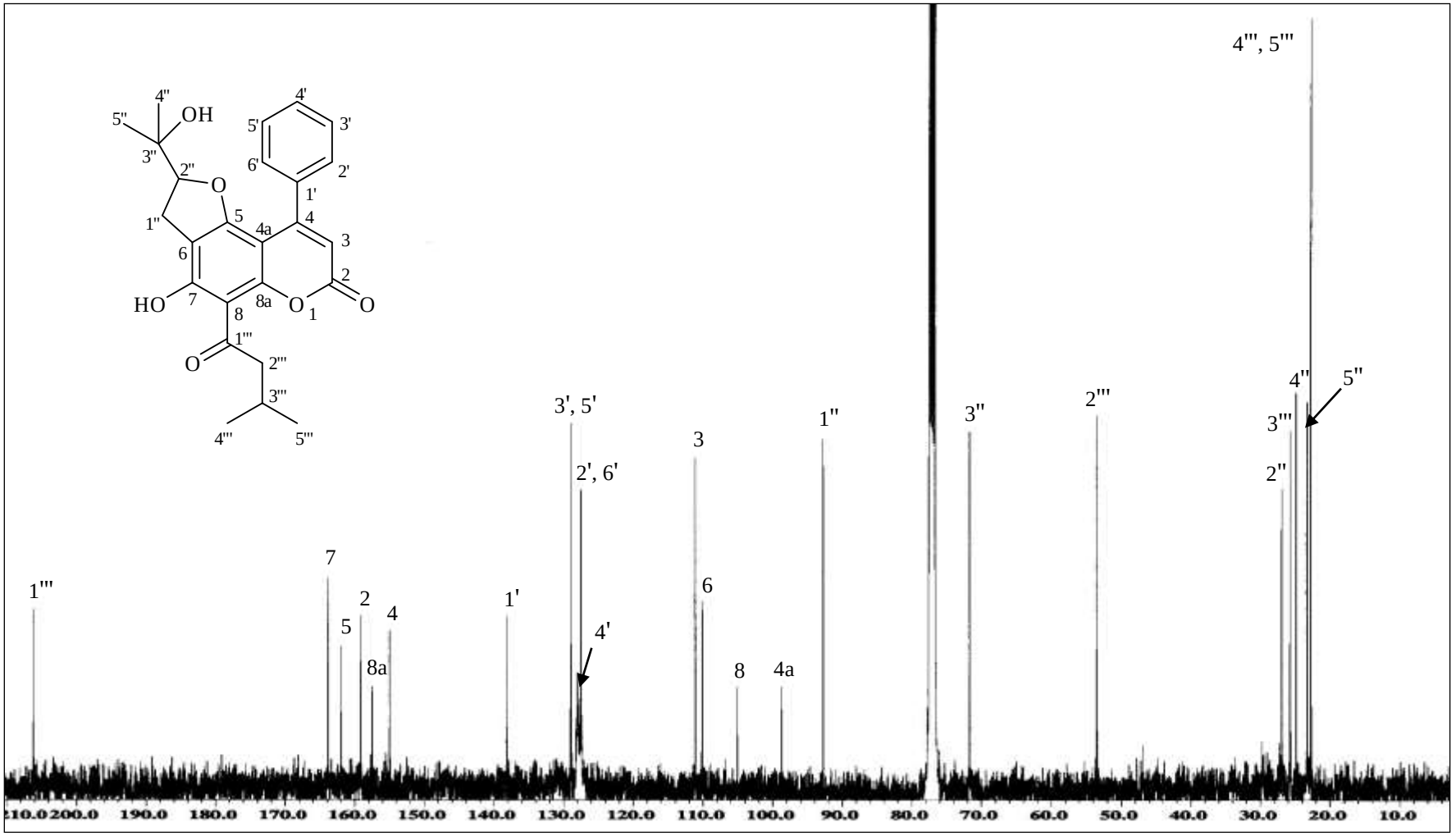
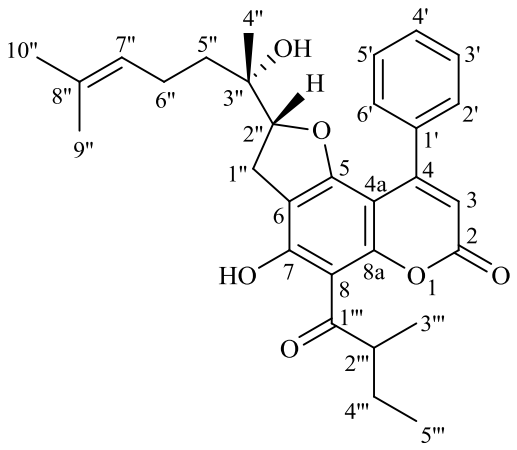


Figure 3.26: ^{13}C NMR Spectrum of Compound M **194**

3.1.14 Compound N: Mesuagenin F 195



195

Separation by using column chromatography, followed by HPLC afforded compound N as a yellow oil with $[\alpha]^{26}_{D}$ +8.9 (*c* 0.0001, EtOH). The HRESIMS spectrum revealed a $[M+Na]^+$ ion at *m/z* 513.2253 (calculated 513.2253), which was associated with the molecular formula of $C_{30}H_{34}O_6$. The UV spectrum (EtOH) supported an 8-acyl-5,7-dioxycoumarin type, with absorptions at λ_{max} 226 and 298 nm[82]. The IR spectrum showed absorptions at υ_{max} 3447 (OH), 1717 (α,β-unsaturated lactone) and 1604 cm^{-1} (chelated acyl group)[47].

The ^{13}C NMR spectrum showed the signals of five methyls, four methylenes, nine methines and twelve quaternary carbons. The ^{1}H NMR spectrum showed the typical singlet of a H-3 proton at δ 6.05. In addition, two sets of multiplets corresponding to five protons centred at δ 7.41 (H-3'-H-5') and 7.29 (H-2', H-6'), respectively were observed, indicating the presence of an unsubstituted phenyl ring. The HMBC spectrum showed correlations of the H-3 proton with δ 138.2 (C-1') and 155.1 (C-4), thus suggesting that the phenyl ring was attached to C-4. A split singlet at δ 14.29 in ^{1}H NMR spectrum can be ascribed to a hydroxyl chelated to an acyl group. All these

observations, in addition with the UV absorptions data, suggested the presence of an 8-acyl-5,7-dioxy-4-phenylcoumarin skeleton[82].

The ^{1}H NMR spectrum of compound N also showed the presence of a triplet at δ 4.53 (1H, *J* = 9.8 Hz, H-2") and two doublets of doublets at 3.04 (1H, *J* = 9.8, 15.9 Hz, H-1"β) and 2.96 (1H, *J* = 9.8, 15.9 Hz, H-1"α) respectively, thus indicating the presence of a dihydrofuran moiety. The ^{1}H NMR resonances at δ 1.24 (2H, *m*, H-5"), 1.97 (2H, *m*, H-6"), 5.03 (1H, br*t*, *J* = 7.3, H-7"), 1.59 (3H, *s*, H-9") and 1.67 (3H, *s*, H-10") together with the ^{13}C signals at δ 36.1 (C-5"), 21.7 (C-6"), 123.9 (C-7"), 132.3 (C-8"), 17.7 (C-9") and 25.8 (C-10") indicated the presence of an isoprenyl group attached to C-3" (δ 73.4). In addition, the HMBC correlations clearly proved that the substituted dihydrofuran ring was located between C-5 (δ 161.9) and C-6 (δ 110.2) by exhibiting the following correlation, H-1"α,β/C-5, C-6, C-2" and C-3".

Signals of non-equivalent geminal protons of C-4"' showed two methylene protons as two sets of multiplets at δ 1.50 (1H, *m*, H-4"'α) and 1.92 (1H, *m*, H-4"'β) in the ^{1}H NMR spectrum. In the HMBC spectrum, these methylene protons correlated with a carbonyl (C-1"', δ 210.5), a methine (C-2"', δ 46.9) and two methyls (C-3"', δ 16.6 and C-5"', δ 11.9). Protons of the two methyls (C-3"' and C-5"') were observed as a doublet at δ 1.26 (*J* = 6.7 Hz) and as a split triplet at δ 1.00 (*J* = 7.4 Hz), respectively. These information led to the conclusion that a 2-methylbutanoyl moiety is linked to C-8 of the coumarin skeleton.

Based on the above data, it is proposed that compound N has the structure shown in **195**. A thorough literature search using the SciFinder database identified compound N as a new chemical entity, with was named as mesuagenin F **195**.

Table 3.15: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound N **195**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	159.2		
3	6.05 (1H, *s*)	111.1		2, 4a, 1'
4	-	155.1		
4a	-	98.8		
5	-	161.9		
6	-	110.2		
7-OH	14.27 (1H, split *s*)	164.0		6, 7, 8
8	-	104.7 (split peaks)		
8a	-	157.2		
1'	-	138.2		
2'	7.30 (1H, *m*, Ar)	127.5		3'
3'	7.41 (3H, *m*, Ar)	128.9		1'
4'		128.0		2', 6'
5'		128.9		6'
6'	7.30 (1H, *m*, Ar)	127.5		5'
1"α	2.96 (1H, *dd*, *J* = 9.8, 15.9)	26.5	H-1"β	5, 6, 2", 3"
1"β	3.04 (1H, *dd*, *J* = 9.8, 15.9)		H-1"α	
2"	4.53 (1H, *t*, *J* = 9.8)	92.3		5"
3"-OH	-	73.4		
4"	0.87 (3H, split *s*)	21.8 (split peaks)		2", 3", 5"
5"	1.24 (2H, *m*)	36.1		2", 3", 4", 6", 7"
6"	1.97 (2H, *m*)	21.7		5", 7", 8"
7"	5.03 (1H, br*t*, *J* = 7.3)	123.9	6"	6", 9", 10"
8"	-	132.3	7"	
9"	1.59 (3H, *s*)	17.7		7", 8", 10"
10"	1.67 (3H, *s*)	25.8		7", 8", 9"
1'"	-	210.5		
2'"	3.94 (1H, *m*)	46.9	3'"	1'", 3'", 4'", 5'"
3'"	1.26 (3H, *d*, *J* = 6.7)	16.6 (split peaks)		1'", 2'", 4'"
4'"α	1.50 (1H, *m*)	27.3 (split peaks)	4'"β, 5'"	1'", 2'", 3'", 5'"
4'"β	1.92 (1H, *m*)		4'"α, 5'"	1'", 2'", 3'", 5'"
5'"	1.00 (3H, split *t*, *J* = 7.4)	11.9		2'", 3'", 4'"

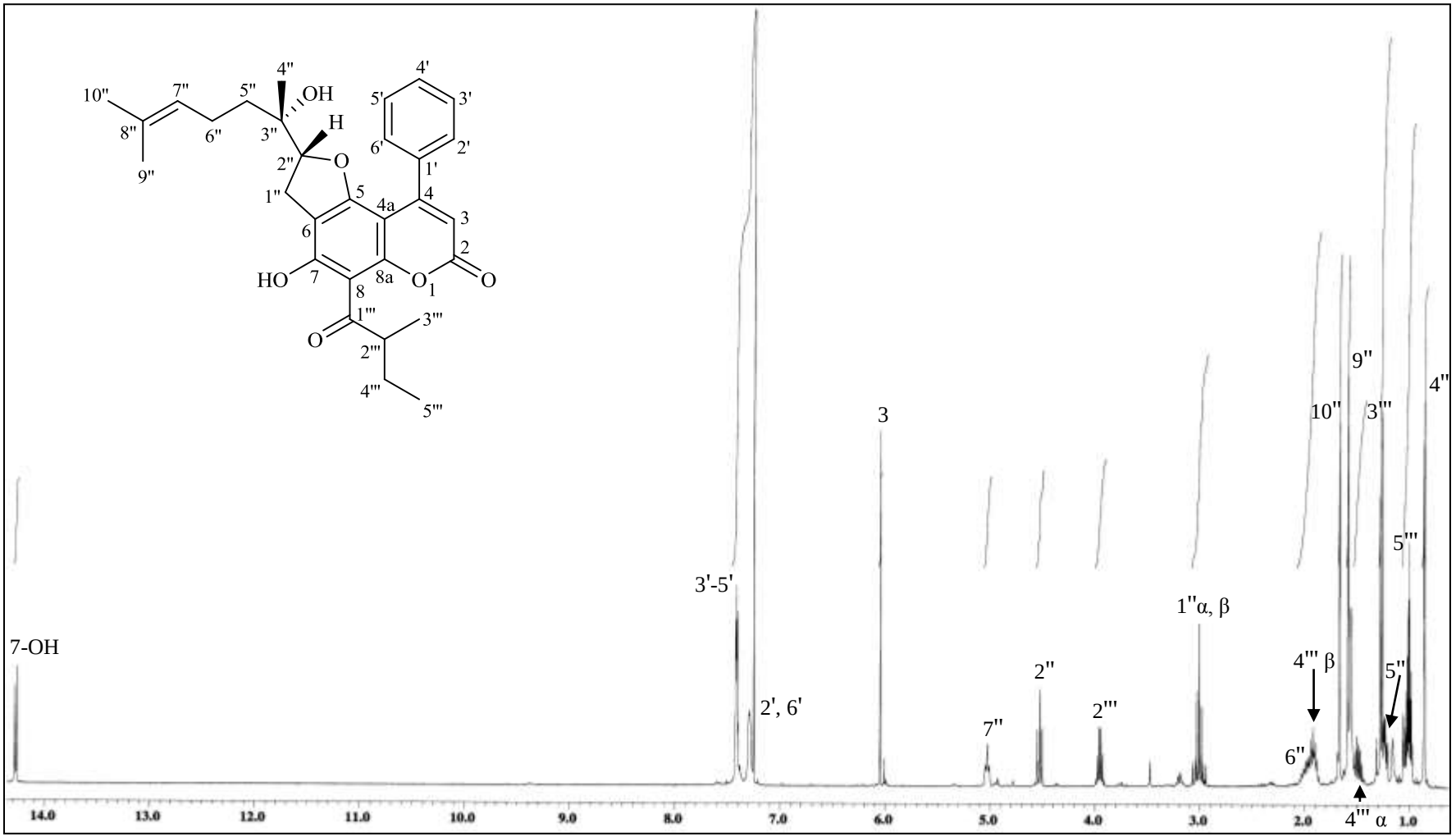


Figure 3.27: ^{1}H NMR Spectrum of Compound N **195**

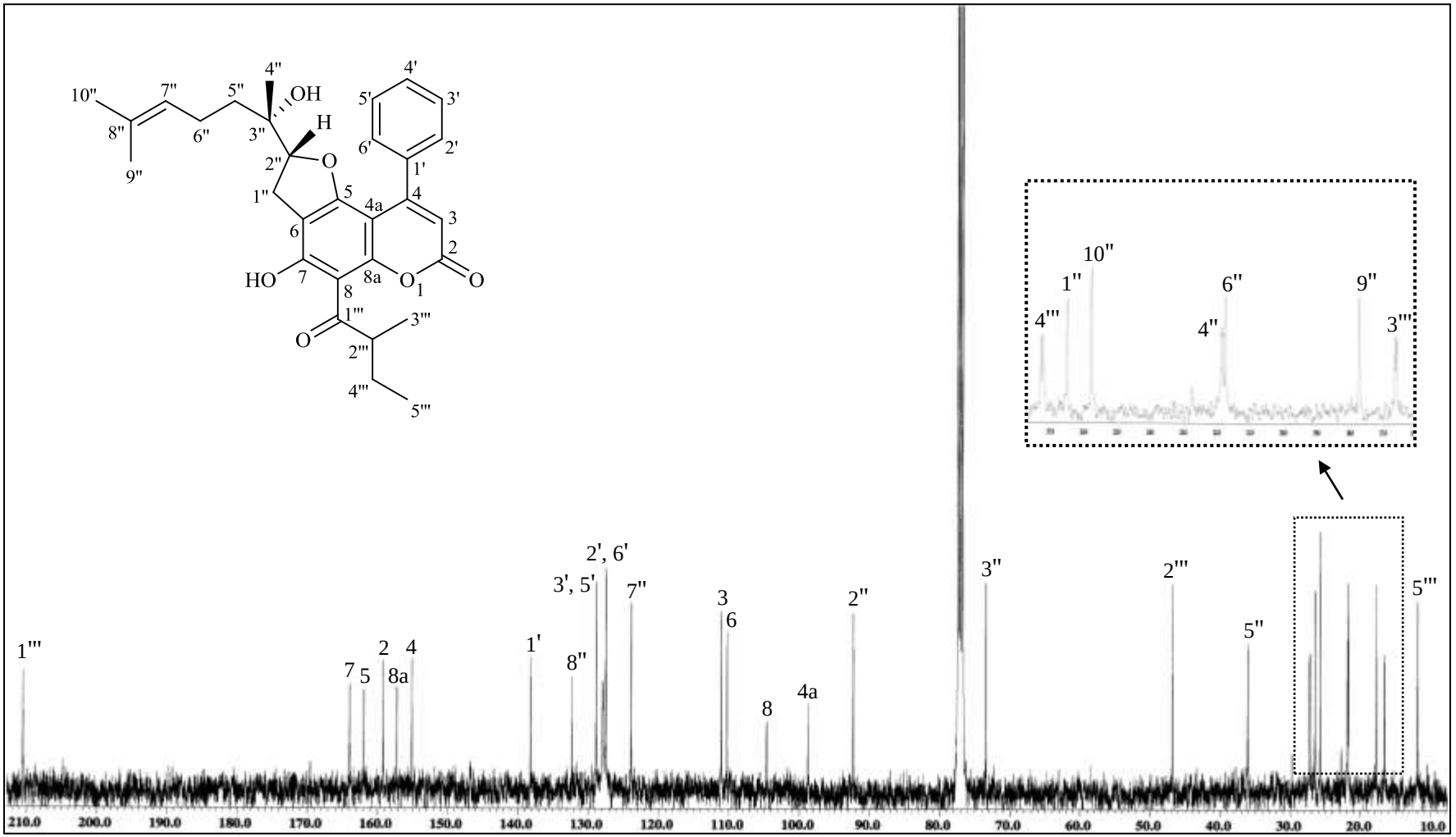
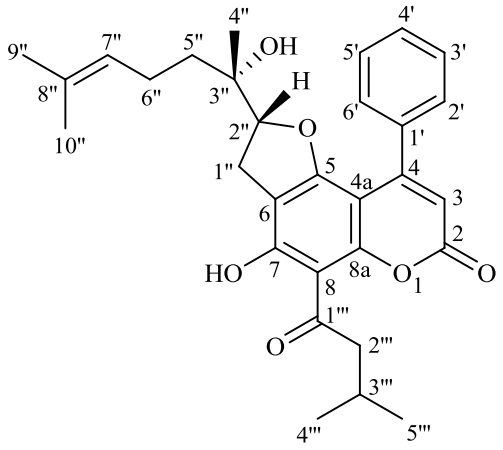


Figure 3.28: ^{13}C NMR Spectrum of Compound N **195**

3.1.15 Compound O: Mesuagenin D 196



196

Compound O was obtained as a yellow oil with $[\alpha]^{26}_{D}$ +7.7 (*c* 0.0001, EtOH), and has a molecular formula of $C_{30}H_{34}O_6$ based on the HRESIMS measurement; a pseudomolecular $[M+Na]^+$ ion at *m/z* 513.2253 (calculated 513.2253). The IR spectrum showed absorptions at υ_{max} 3445 (OH), 1718 (α,β-unsaturated lactone), 1603 (chelated acyl group) and 1385 cm^{-1} (geminal dimethyl)[47]. The UV spectrum (EtOH) supported an 8-acyl-5,7-dioxycoumarin type, with absorptions at λ_{max} 226 and 298 nm[82].

The ^{13}C NMR spectrum of compound O displayed a total of thirty carbons; five methyls, four methylenes, nine methines and twelve quaternary carbons. The ^{1}H and ^{13}C NMR spectra of compounds O and N showed similar patterns, which suggested a close structural relationship between these compounds. Indeed, the same substituents for the coumarin skeleton could be characterized in both compounds; a typical H-3 singlet of a 4-phenylcoumarin (δ 6.04, *s*, H-3), a monosubstituted phenyl (δ 7.42 and 7.30, 5H, *m*, H-2'-H6'), a furan-cyclized geranyl group [δ 3.09 (1H, *dd*, *J* = 9.8, 15.9 Hz, H-1"α), 2.49 (1H, *dd*, *J* = 9.8, 15.9 Hz, H-1"β), 4.53 (1H, *t*, *J* = 9.8 Hz, H-2"), 0.87 (3H, *s*, H-4"), 1.24 (2H, *dd*, *J* = 7.8, 16.6 Hz, H-5"), 1.94 (2H, *m*, H-6"), 5.01 (1H, br*t*, *J* = 7.3 Hz, H-

7''), 1.59 (3H, *s*, H-9'') and 1.67 (3H, *s*, H-10'')] and a chelated hydroxyl (δ 14.29, *s*, 7-OH).

However, close analysis of the ^{1}H and ^{13}C NMR spectra of compound O showed evident differences in the coupling patterns of the substituent group at position C-8 as compared to compound N. The cross correlations detected from the COSY spectrum of compound O revealed the presence of an *iso*-butyryl spin system with signals at δ 3.16 (2H, *d*, *J* = 6.7 Hz, H-2'''), 2.30 (1H, *m*, H-3''') and 1.04 (6H, *d*, *J* = 7.3 Hz, H-4''' and H-5'''). In the HMBC spectrum of compound O, the H-2''' methylene protons caused cross-peaks with C-8 at δ 105.06 and a keto function at δ 206.13 (C-1'''). This information led to the deduction that a 3-methylbutanoyl chain is the substituent at C-8.

Based on the above observed data, it was determined that compound O has the structure as shown **196**. A thorough literature search using the SciFinder database identified compound O as a new chemical entity, with the IUPAC name 7-hydroxy-8-(3-methylbutanoyl)-6-(1-hydroxy-1-methylprenyl)-4-phenyl-2*H*-furo[2',3':3,4]benzo[1,2-*b*]pyran-2-one **196**, which was named as mesuagenin D[92].

Table 3.16: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound O **196**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	159.2		
3	6.04 (1H, *s*)	111.1		2, 4a, 1'
4	-	155.0		
4a	-	98.8		
5	-	162.0		
6	-	110.2		
7-OH	14.29 (1H, *s*)	163.8		6, 7, 8
8	-	105.1		
8a	-	157.4		
1'	-	138.2		
2'	7.30 (1H, *m*, Ar)	127.5		3'
3'	7.42 (3H, *m*, Ar)	128.9		1'
4'		128.0		2'
5'		128.9		
6'	7.30 (1H, *m*, Ar)	127.5		
1"α	3.09 (1H, *dd*, *J* = 9.8, 15.9)	26.5	2"	5, 6, 2", 4"
1"β	2.49 (1H, *dd*, *J* = 9.8, 15.9)		2"	5, 6, 2", 4"
2"	4.53 (1H, *t*, *J* = 9.8)	92.4	1"α, 1"β	
3"-OH	-	73.4		
4"	0.87 (3H, *s*)	21.9		1", 3", 5"
5"	1.24 (2H, *dd*, *J* = 7.8, 16.6)	36.1		1", 3", 6"
6"	1.94 (2H, *m*)	21.8		5", 7", 8"
7"	5.01 (1H, *brt*, *J* = 7.3)	123.9	6"	6", 9", 10"
8"	-	132.3	7"	
9"	1.59 (3H, *s*)	17.7		7", 8", 10"
10"	1.67 (3H, *s*)	25.8		7", 8", 9"
1"'	-	206.1		
2"'	3.16 (2H, *d*, *J* = 6.7)	53.5	3"'	8, 1"', 3"', 4"', 5"'
3"'	2.30 (1H, *m*)	25.7	4"', 5"'	4"', 5"'
4"'	1.04 (6H, *d*, *J* = 7.3)	22.8	3"', 5"'	2"', 3"', 5"'
5"'		22.8	3"', 4"'	2"', 3"', 4"'

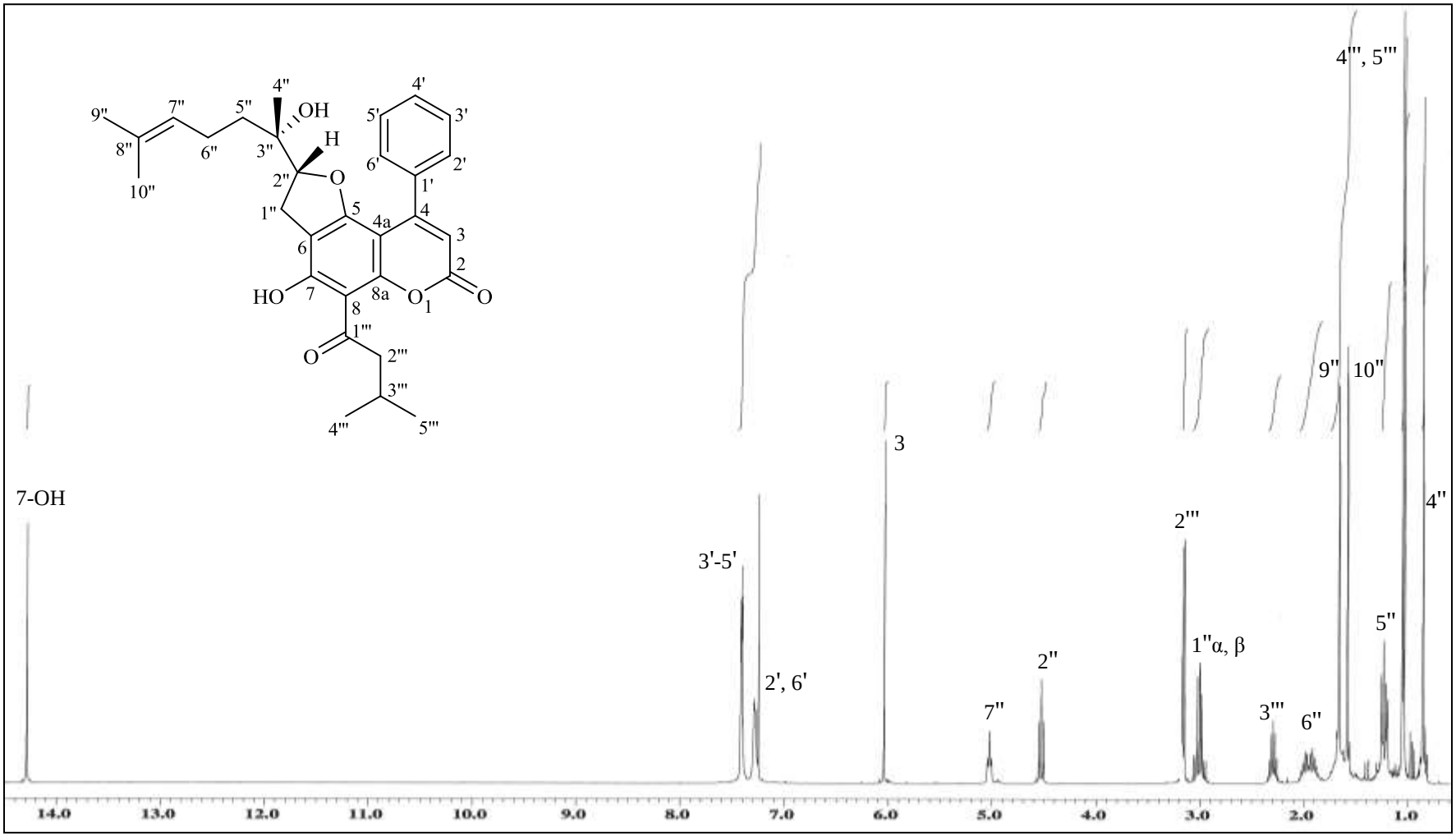


Figure 3.29: ^{1}H NMR Spectrum of Compound O **196**

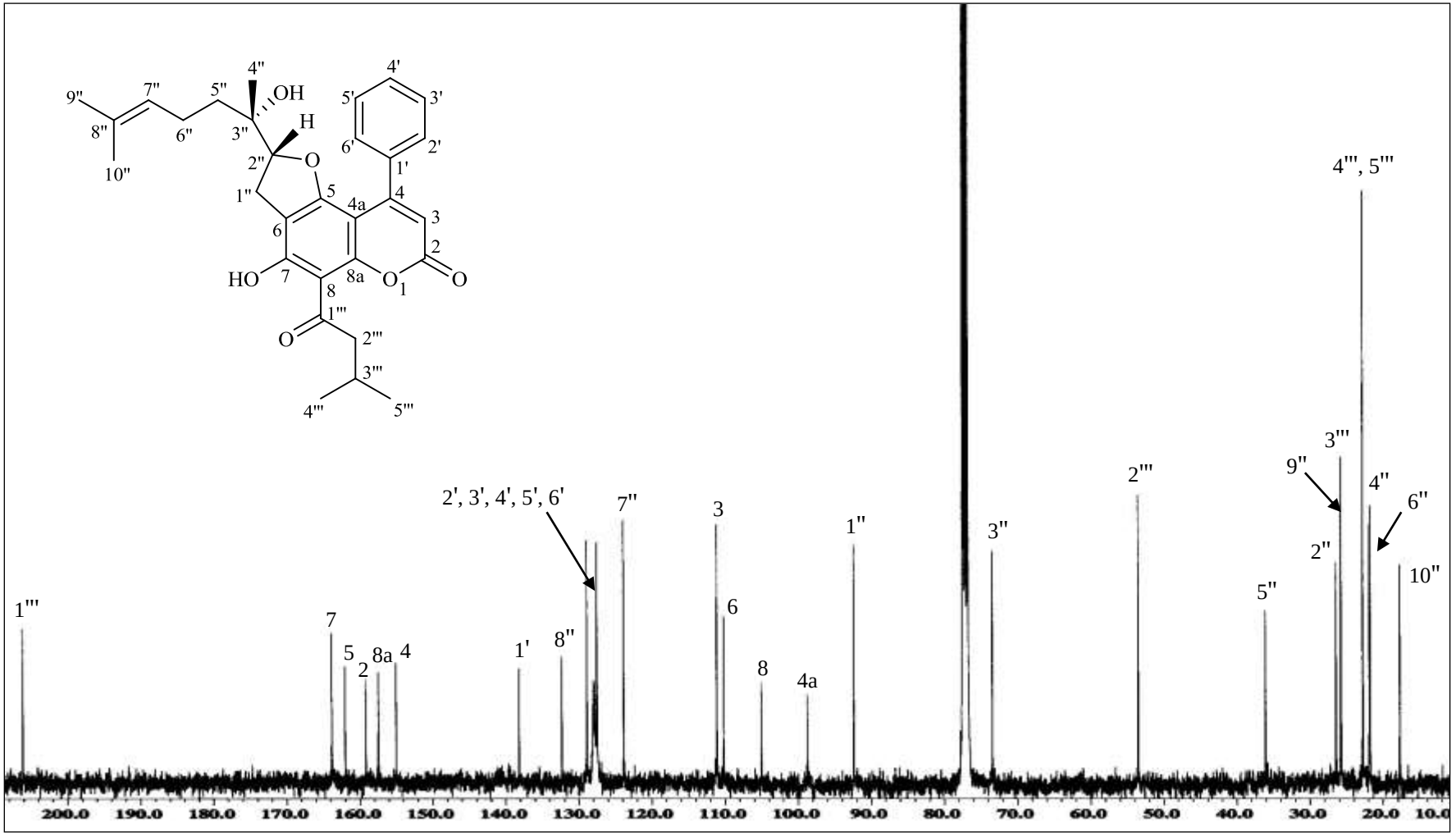
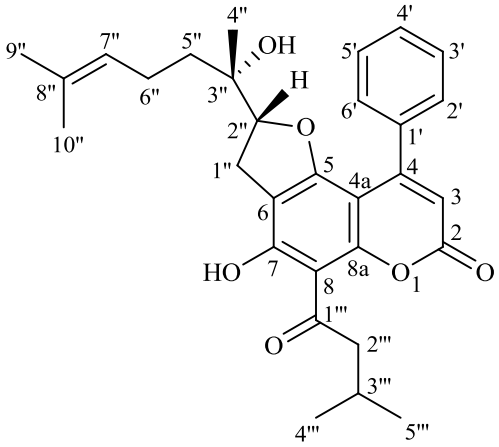


Figure 3.30: ^{13}C NMR Spectrum of Compound O **196**



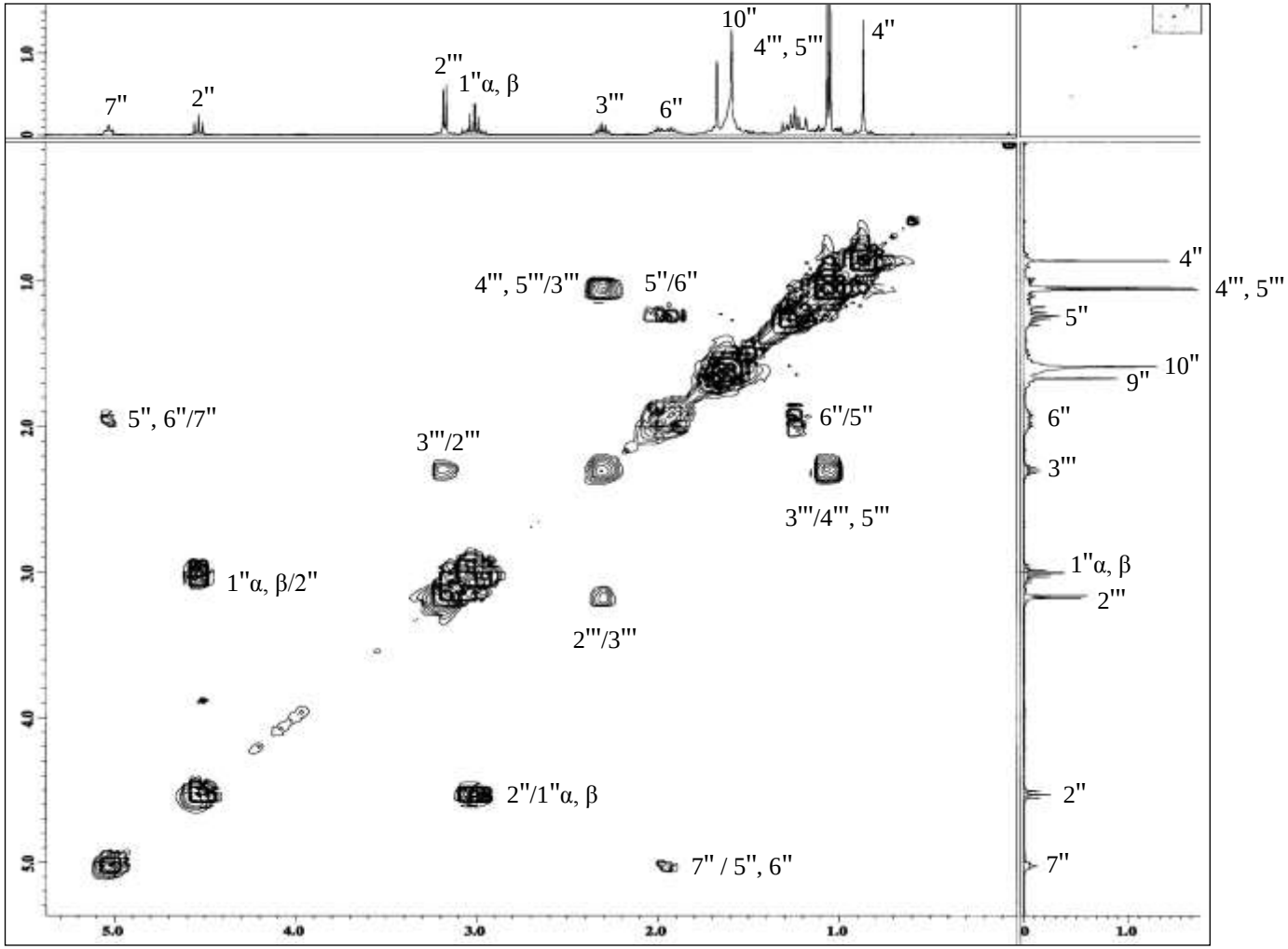
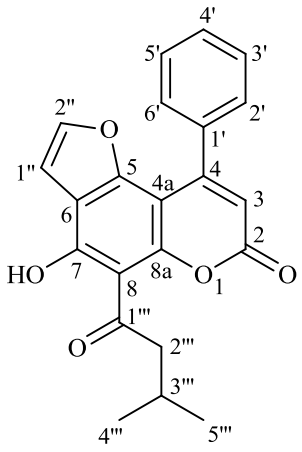


Figure 3.31: COSY Spectrum of Compound O **196**

3.1.16 Compound P: Isodisparfuran 197



197

Separation using column chromatography, followed by HPLC, afforded compound P as a white amorphous solid. The HRESIMS measurement of its $[M+Na]^+$ ion at *m/z* 385.1057 (calculated 385.1052) corresponded to the molecular formula of $C_{22}H_{18}O_5$. The UV spectrum of compound P could readily be associated with an 8-acyl-5,7-dioxycoumarin type, due to absorptions at λ_{max} 230, 287 and 337 nm[82]. The IR spectrum showed absorptions at υ_{max} 1736 cm^{-1}, indicating the presence of an α-pyrone group. The IR spectrum also exhibited absorptions at υ_{max} 1605 and 1383 cm^{-1}, corroborating the presence of a chelated acyl group and a geminal dimethyl group[47, 85].

The ^{13}C NMR spectrum of compound P showed twenty-two carbon resonances; two methyls, one methylene, nine methines and ten quaternary carbons. In the ^1H NMR spectrum of compound P, the typical H-3 singlet of a 4-substituted coumarin was observed at δ 6.22. This characteristic singlet of H-3 was further corroborated with the presence of two sets of multiplets, centered at δ 7.50 (H-3' - H-5') and 7.42 (H-2' and H-6'), which corresponded to two and three aromatic protons of the monosubstituted phenyl ring attached to C-4. The ^1H NMR spectrum of compound P also revealed the presence of a chelated hydroxyl group at δ 14.86, which was exchangeable with D_2O.

All the observations supported the presence of an 8-acyl-5,7-dioxy-4-phenylcoumarin type skeleton.

Two distinct olefinic protons were apparent in the ^{1}H NMR of compound P as two doublets; δ 7.32 (1H, *J* = 1.4 Hz, H-2") and 6.96 (1H, *J* = 1.4 Hz, H-1"). H-2" was more deshielded than H-1", due to the presence of a more electronegative oxygen atom adjacent to C-2". These olefinic protons correlated with C-5 (δ 153.7) and C-6 (δ 115.0) in the HMBC spectrum of compound P, suggesting a two-carbon substituent at position C-5 and C-6.

The cross correlation of the COSY spectrum of compound P revealed an *iso*-butyryl spin system, with signals at δ 3.29 (2H, *d*, *J* = 6.7 Hz, H-2'"), δ 2.37 (1H, *m*, H-3'") and δ 1.08 (6H, *d*, *J* = 6.7 Hz, H-4'" and H-5'"). Furthermore, in the HMBC spectrum, the methylene protons gave cross-peaks with a keto function at δ 207.3 (C-1'"). These observations in the COSY and HMBC spectra of compound P led to the identification of the remaining substituent of C-8 of the 4-phenylcoumarin skeleton as a 3-methylbutanoyl chain.

The observed data of compound P indicated that this compound was isodisparfuran A **197**, as reported in the literature, and was first isolated from *Calophyllum dispar* (Clusiaceae) in 2001 by Guilet *et al.*[85].

Table 3.17: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound P **197**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	159.2		
3	6.22 (1H, *s*)	112.0		2, 4a, 1'
4	-	154.2		
4a	-	100.0		
5	-	153.7		
6	-	115.0		
7-OH	14.86 (1H, *s*)	162.7		6, 7, 8
8	-	106.1		
8a	-	154.5		
1'	-	137.1		
2'	7.42 (1H, *m*, Ar)	127.9		4, 4'
3'	7.50 (3H, *m*, Ar)	128.1		1', 2', 6'
4'		129.4		
5'		128.1		
6'	7.42 (1H, *m*, Ar)	127.9		4, 4'
1"	6.96 (1H, *d*, *J* = 1.4)	104.9	1"	5, 6, 2"
2"	7.32 (1H, *d*, *J* = 1.4)	144.6	2"	5, 6, 1"
1'"	-	207.3		
2'"	3.29 (2H, *d*, *J* = 6.7)	53.7	3'"	1'", 3'", 4'", 5'"
3'"	2.37 (1H, *m*)	25.6	2'", 4'", 5'"	2'", 4'", 5'"
4'"	1.08 (6H, *d*, *J* = 6.7)	22.7	5'"	2'", 3'", 5'"
5'"		22.7	4'"	2'", 3'", 4'"

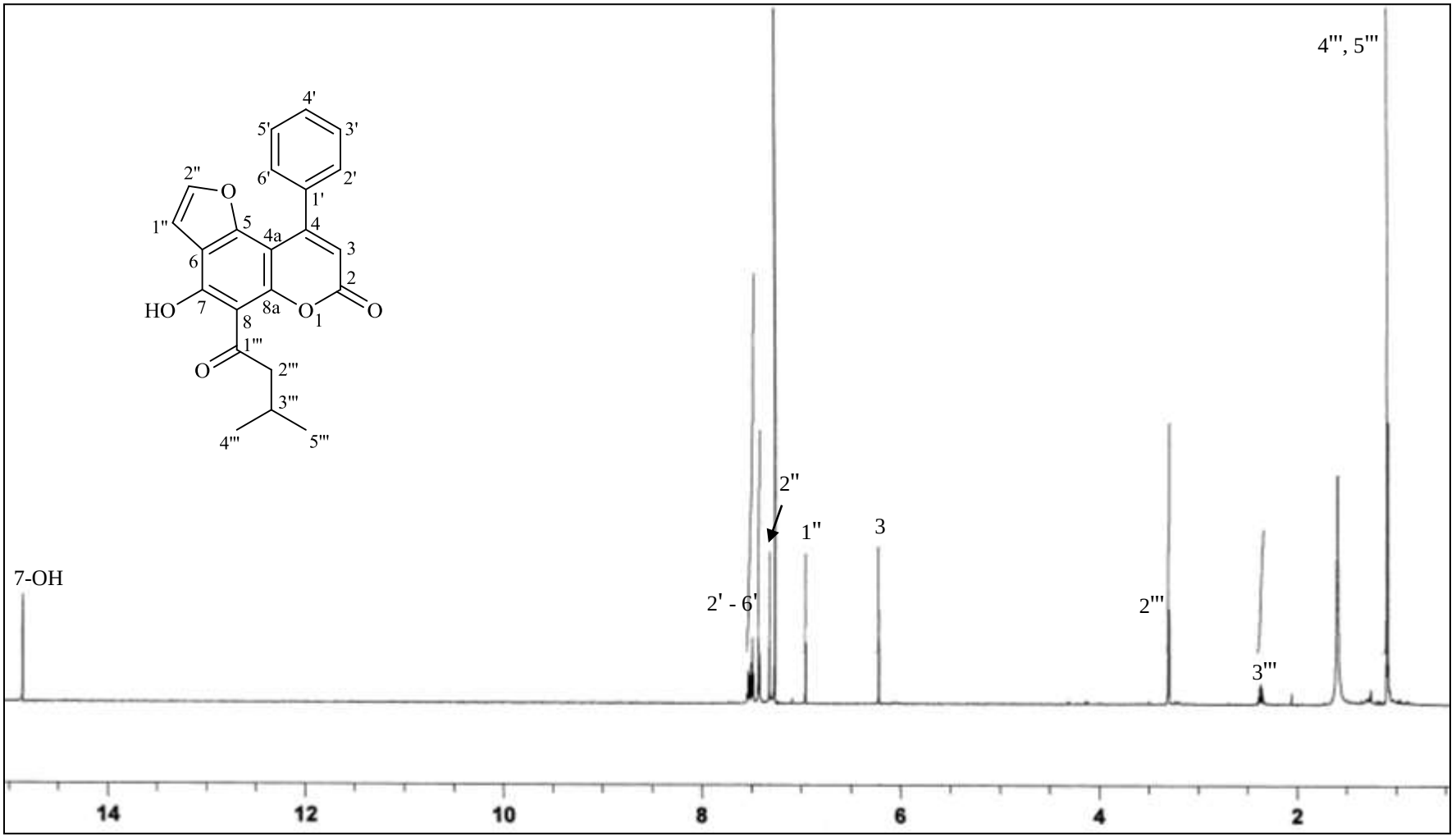


Figure 3.32: ^{1}H NMR Spectrum of Compound P **197**

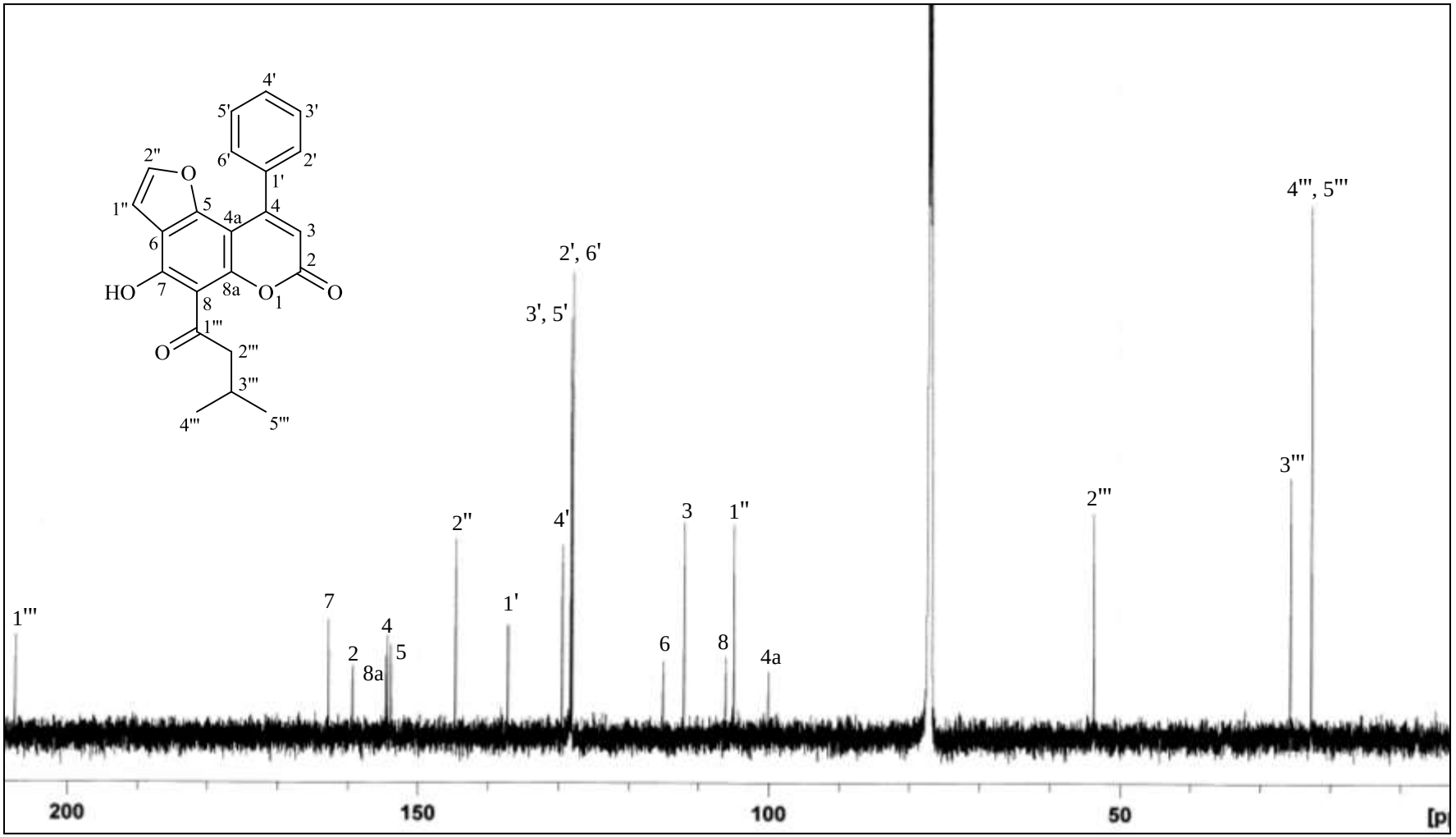


Figure 3.33: ^{13}C NMR Spectrum of Compound P **197**

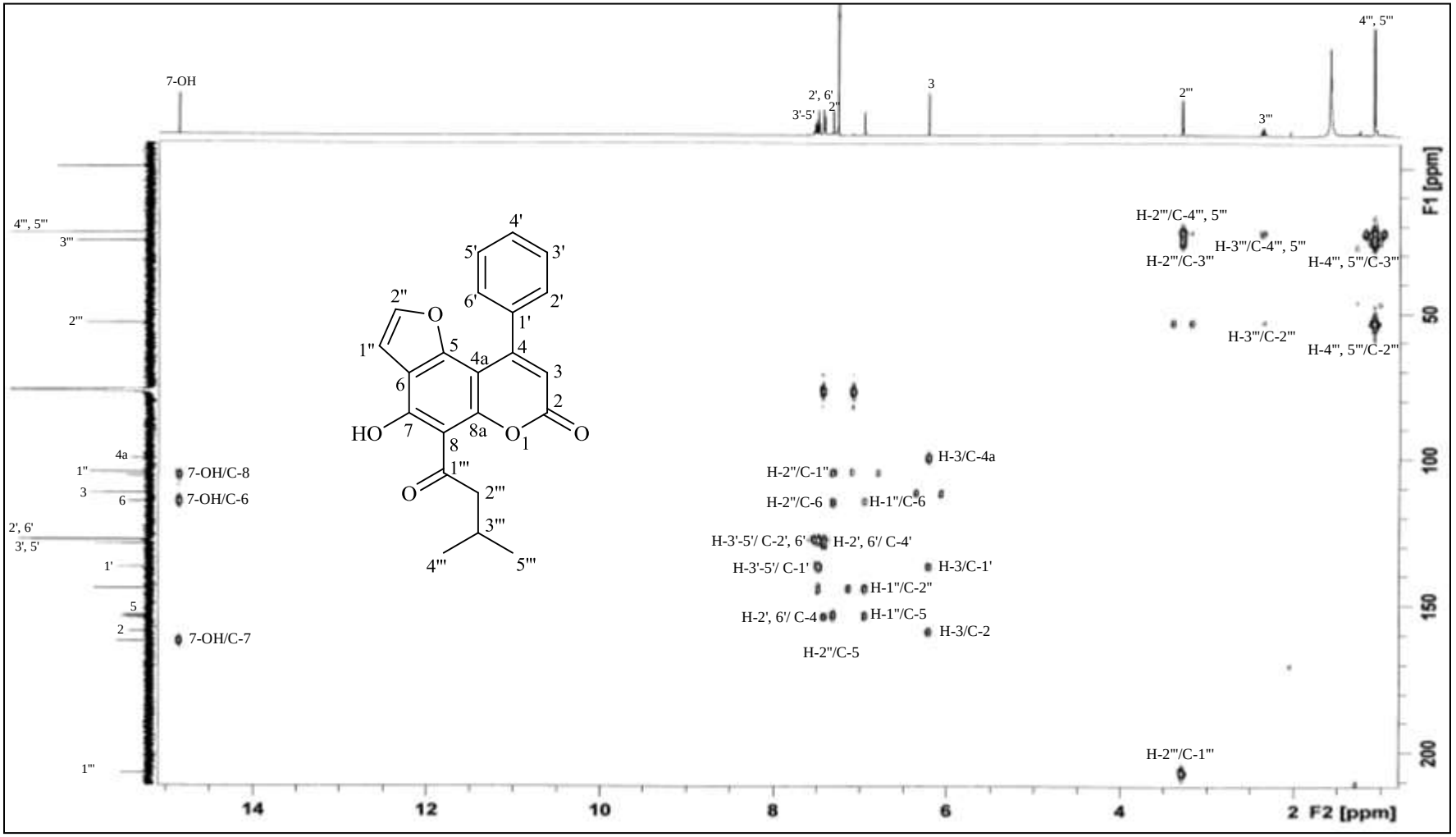
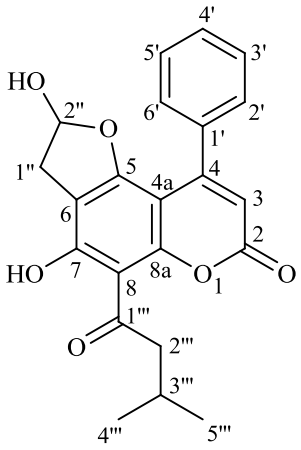


Figure 3.34: HMBC Spectrum of Compound P **197**

3.1.17 Compound Q: Mesuagenin G 198



198

Compound Q was obtained as a white amorphous solid, and the HRESIMS measurement of its $[M+Na]^+$ ion at *m/z* 403.1163 (calculated 403.1158) suggested a molecular formula of $C_{22}H_{20}O_6$. The UV spectrum supported an 8-acyl-5,7-dioxycoumarin type, with absorptions at λ_{max} 230, 287 and 337 nm[82]. The IR spectrum showed absorptions at υ_{max} 3451 (OH), 1744 (α,β-unsaturated lactone) and 1605 cm^{-1} (chelated acyl group)[47, 85].

The ^{13}C NMR spectrum of compound Q displayed a total of twenty-two carbons; two methyls, two methylenes, eight methines and ten quaternary carbons. The ^{1}H NMR spectrum of compound Q showed striking similarities with the ^{1}H NMR spectrum of compound P, with similar substituents observed at positions C-4 (monosubstituted phenyl; δ 7.40 and 7.34, 5H, *m*, H-2'-H6'), C-7 (chelated hydroxyl group; δ 14.26, *s*, 7-OH) and C-8 [3-methylbutanoyl; δ 3.17 (2H, *d*, *J* = 6.7 Hz, H-2'''), 2.30 (1H, *m*, H-3''') and 1.04 (6H, *d*, *J* = 6.7 Hz, H-4''' and H-5''')].

Close inspection of the ^{1}H and ^{13}C NMR spectra of compound Q showed a marked difference in the coupling patterns of the substituent groups at positions C-5 and C-6 as

compared to compound P. The sp^2 carbons C-1" and C-2" in compound P were replaced with an sp^3 methine at δ 33.5 and a deshielded sp^3 methylene at δ 103.5 in the ^{13}C spectrum of compound Q. The methylene protons of C-1" appeared as a set of two broad doublets at δ 2.95 (H-1"α) and 3.19 (H-1"β). This suggests that C-2" is an oxymethine and the corresponding protons, H-2" and 2"-OH resonated at δ 5.96 and 3.63, as broad singlets, respectively. These observations showed that the double bond of C-5 and C-6 in the furan ring has been hydroxylated.

Based on the thorough search through SciFinder database, it was found that compound Q was a new chemical entity, with the IUPAC name 2,4-dihydroxy-5-(3-methylbutanoyl)-9-phenyl-2*H*-furo[2,3-*f*]chromen-7(3*H*)-one, which was named as mesuagenin **198**.

Table 3.18: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound Q **198**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	159.2		
3	6.05 (1H, *s*)	111.6		2, 4a, 1'
4	-	155.1		
4a	-	98.8		
5	-	153.7		
6	-	108.4		
7-OH	14.26 (1H, *s*)	163.8		6, 7, 8
8	-	105.4		
8a	-	157.6		
1'	-	137.7		
2'	7.34 (1H, *m*, Ar)	127.8		4, 4'
3'	7.40 (3H, *m*, Ar)	128.1		
4'		129.0		2', 6'
5'		128.1		
6'	7.34 (1H, *m*, Ar)	127.8		4, 4'
1"α	2.95 (1H, br*d*)	33.5	1"β	2", 5, 6
1"β	3.19 (1H, br*d*)		1"α	5, 6, 7
2"	5.96 (1H, br*s*)	103.5	1"β	
2"-OH	3.63 (1H, br*s*)			
1"'	-	206.3		
2"'	3.17 (2H, *d*, *J* = 6.7)	53.6	3"'	1"', 3"', 4"', 5"'
3"'	2.30 (1H, *m*)	25.7	2"', 4"', 5"'	2"', 4"', 5"'
4"'	1.04 (6H, *d*, *J* = 6.7)	22.8	3"', 5"'	2"', 3"', 5"'
5"'		22.8	3"', 4"'	2"', 3"', 4"'

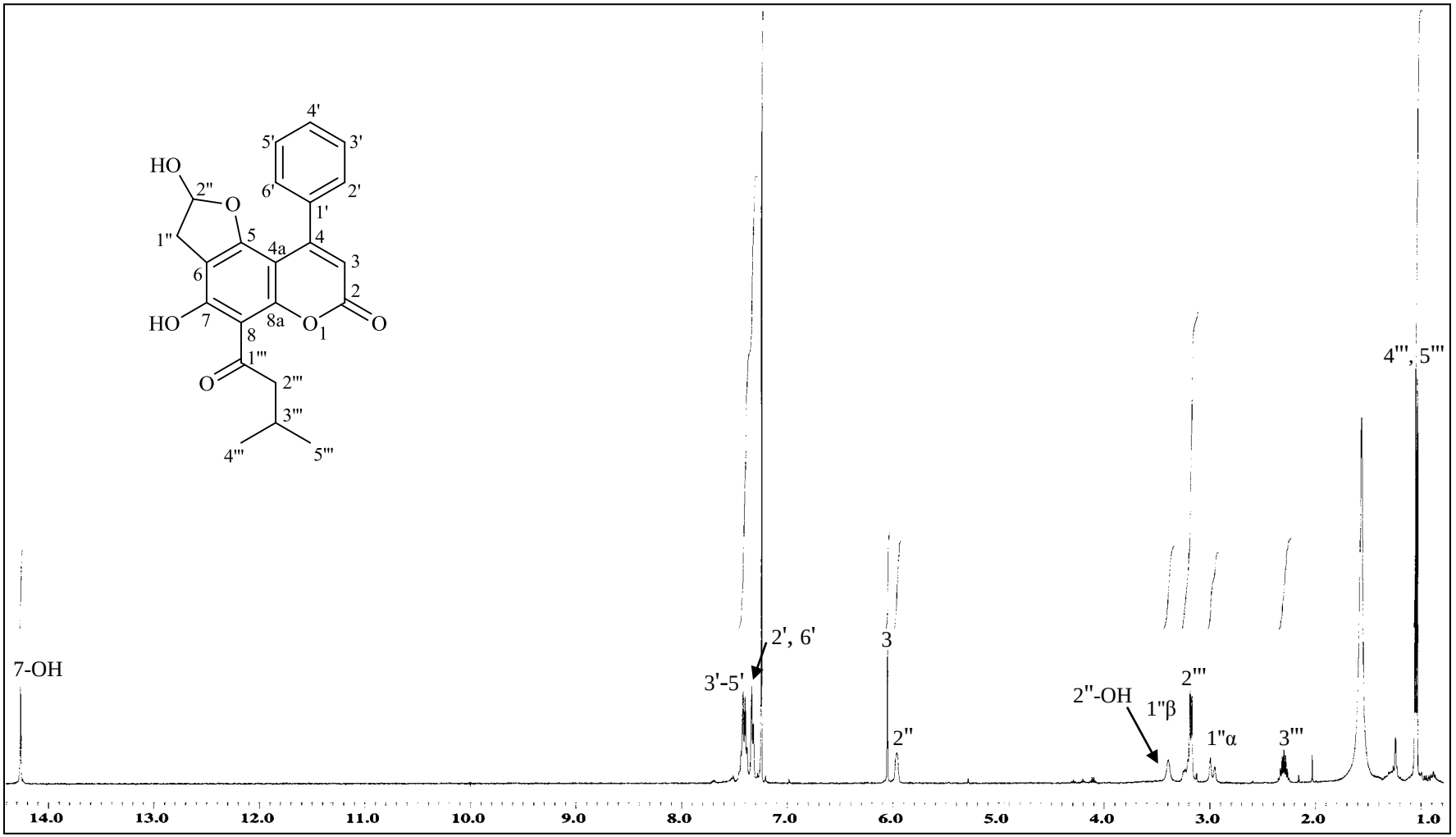


Figure 3.35: ^{1}H NMR Spectrum of Compound Q **198**

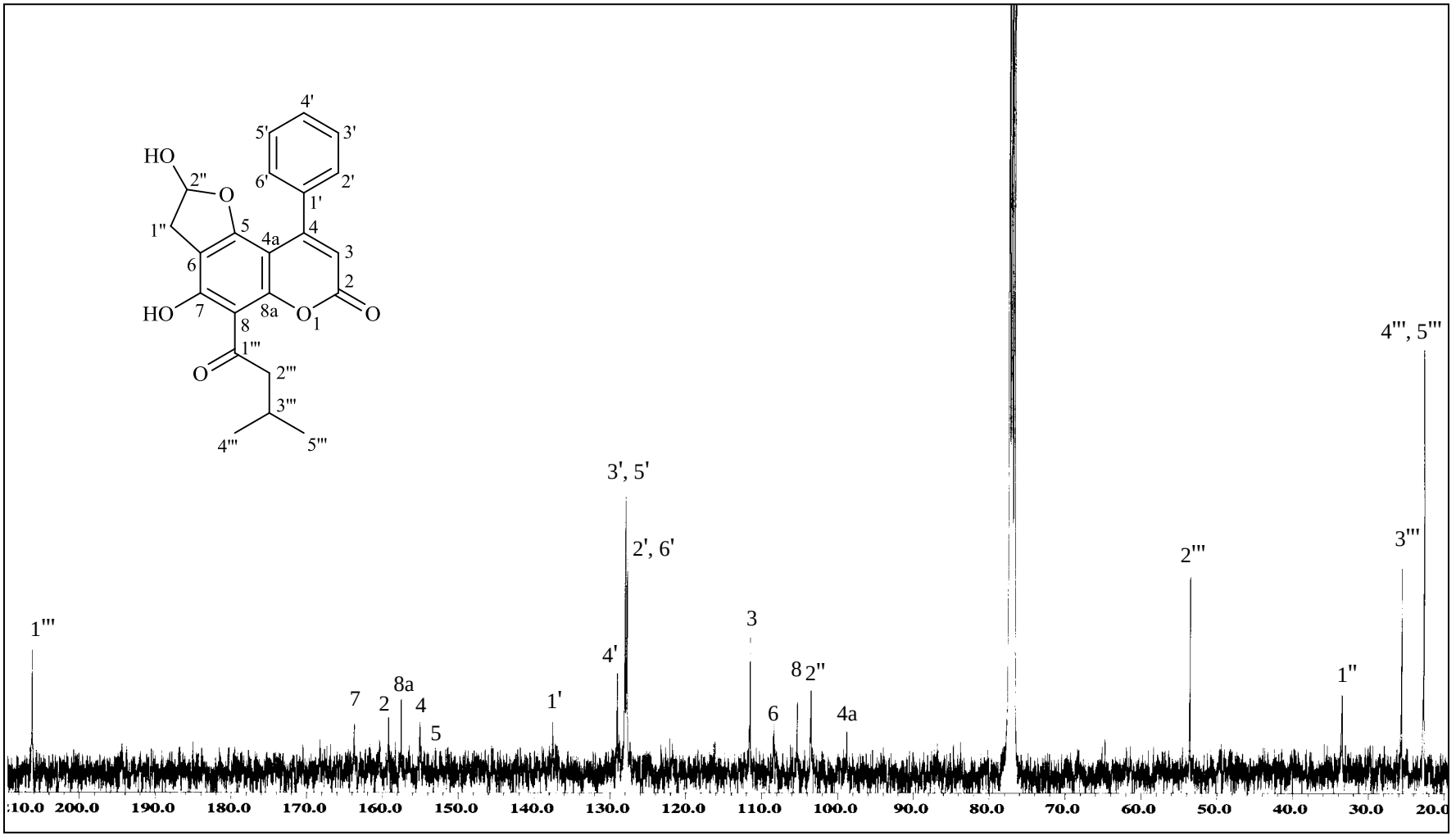


Figure 3.36: ^{13}C NMR Spectrum of Compound Q **198**

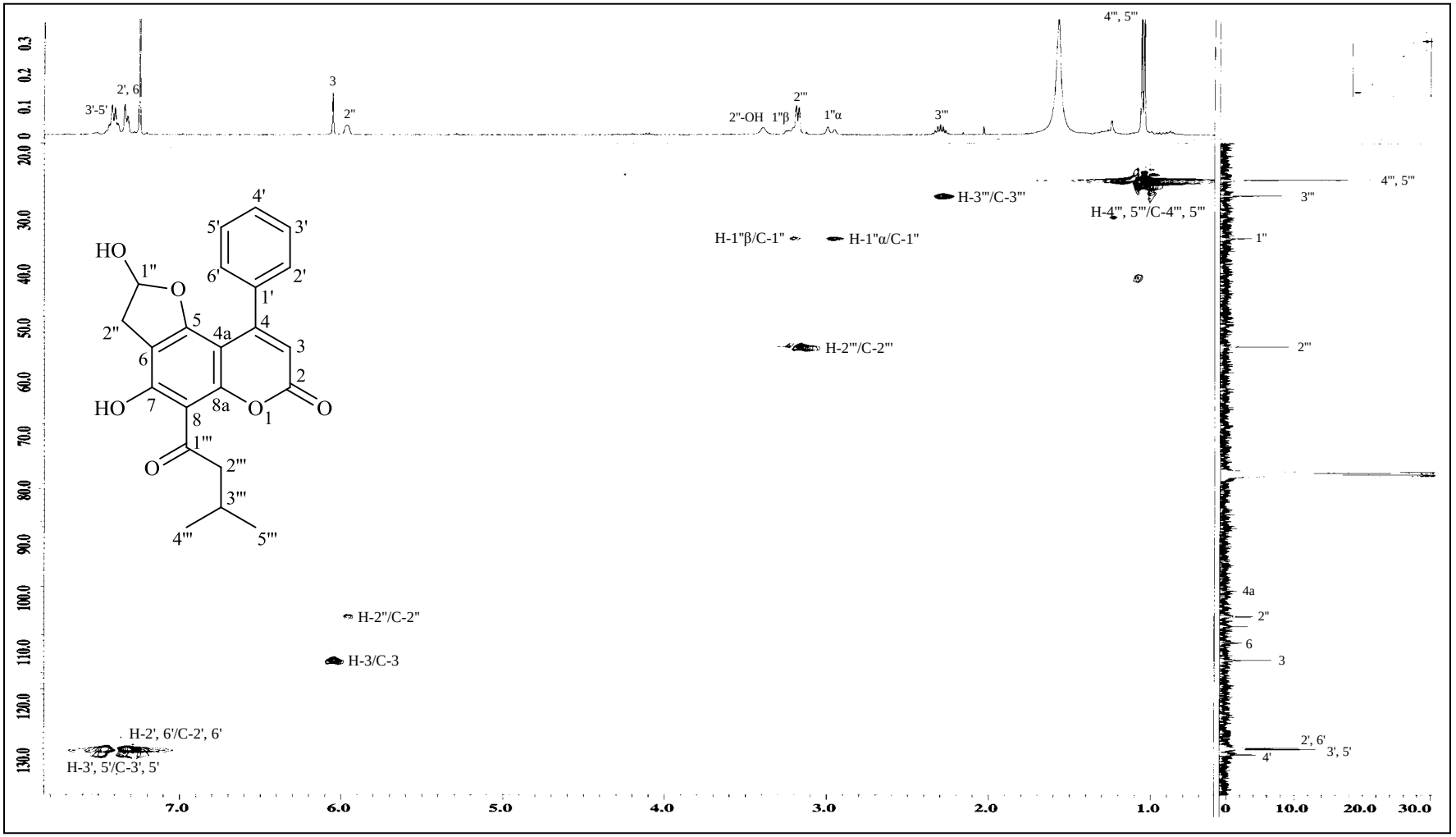
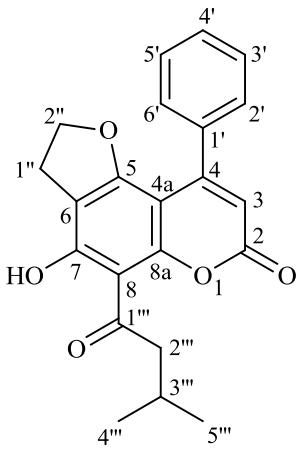


Figure 3.37: HSQC Spectrum of Compound Q **198**

3.1.18 Compound R: Mesuagenin H 199



199

Compound R was obtained as a white amorphous solid using column chromatography fractionation, followed by HPLC separation. The HRESIMS measurement of its $[M+Na]^+$ ion at *m/z* 387.1204 (calculated 387.1208) suggested a molecular formula of $C_{22}H_{20}O_5$. The UV spectrum supported an 8-acyl-5,7-dioxycoumarin type, with absorptions at λ_{max} 230, 285 and 335 nm[82]. The IR spectrum showed absorptions at υ_{max} 1740 (α,β-unsaturated lactone) and 1603 cm^{-1} (chelated acyl group)[47, 85].

A total of twenty-two carbons were observed in the ^{13}C NMR spectrum of compound R; two methyls, three methylenes, seven methines and ten quaternary carbons. The ^1H NMR spectrum of compound R was similar to that of compound P, thus suggesting a close relationship between these compounds. Indeed, the same substituents were characterized in both compounds, namely a monosubstituted phenyl (δ 7.32 and 7.42, 5H, *m*, H-2'-H6'), a chelated hydroxyl group (δ 14.79, *s*, 7-OH) and a 3-methylbutanoyl [δ 3.19 (2H, *d*, *J* = 6.7 Hz, H-2'''), δ 2.31 (1H, *m*, H-3''') and δ 1.05 (6H, *d*, *J* = 6.7 Hz, H-4''' and H-5''').

However, close examination of the ^{1}H NMR spectrum of compound R showed a noticeable difference in the coupling pattern of the substituent groups at positions C-5 and C-6 as compared to compound P. This observation was made clear in the ^{1}H NMR spectrum of compound R, with methylene peaks observed at δ 2.93 (*t*, *J* = 5.5 Hz, H-1") and δ 3.89 (*t*, *J* = 5.5 Hz, H-2"). The furan ring at position C-5 and C-6 in compound R has been hydrogenated as compared to compound P, which possessed an unsaturated furan ring.

A careful search through SciFinder database led to the conclusion that compound R was a new chemical entity, with the IUPAC name 2,4-dihydroxy-5-(3-methylbutanoyl)-9-phenyl-2*H*-furo[2,3-*f*]chromen-7(3*H*)-one, which was named as mesuagenin H **199**.

Table 3.19: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound R **199**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	159.2		
3	6.02 (1H, *s*)	112.3		2, 4a, 1'
4	-	156.2		
4a	-	102.1		
5	-	160.2		
6	-	111.0		
7-OH	14.79 (1H, *s*)	166.8		6, 7, 8
8	-	104.3		
8a	-	156.6		
1'	-	139.5		
2'	7.32 (1H, *m*, Ar)	127.2		4, 5'
3'	7.42 (3H, *m*, Ar)	128.1		1', 6'
4'		128.6		1', 2', 6'
5'		128.1		1', 2'
6'	7.32 (1H, *m*, Ar)	127.2		3', 4'
1"	2.93 (2H, *t*, *J* = 5.5)	25.3	2"	5, 6, 7, 2"
2"	3.89 (2H, *t*, *J* = 5.5)	64.1	1"	6
1'"	-	206.2		
2'"	3.19 (2H, *d*, *J* = 6.7)	53.6	3'"	1'", 3'", 4'", 5'"
3'"	2.31 (1H, *m*)	25.8	2'", 4'", 5'"	2'", 4'", 5'"
4'"	1.05 (6H, *d*, *J* = 6.7)	22.8	3'"	2'", 3'", 5'"
5'"		22.8	3'"	2'", 3'", 4'"

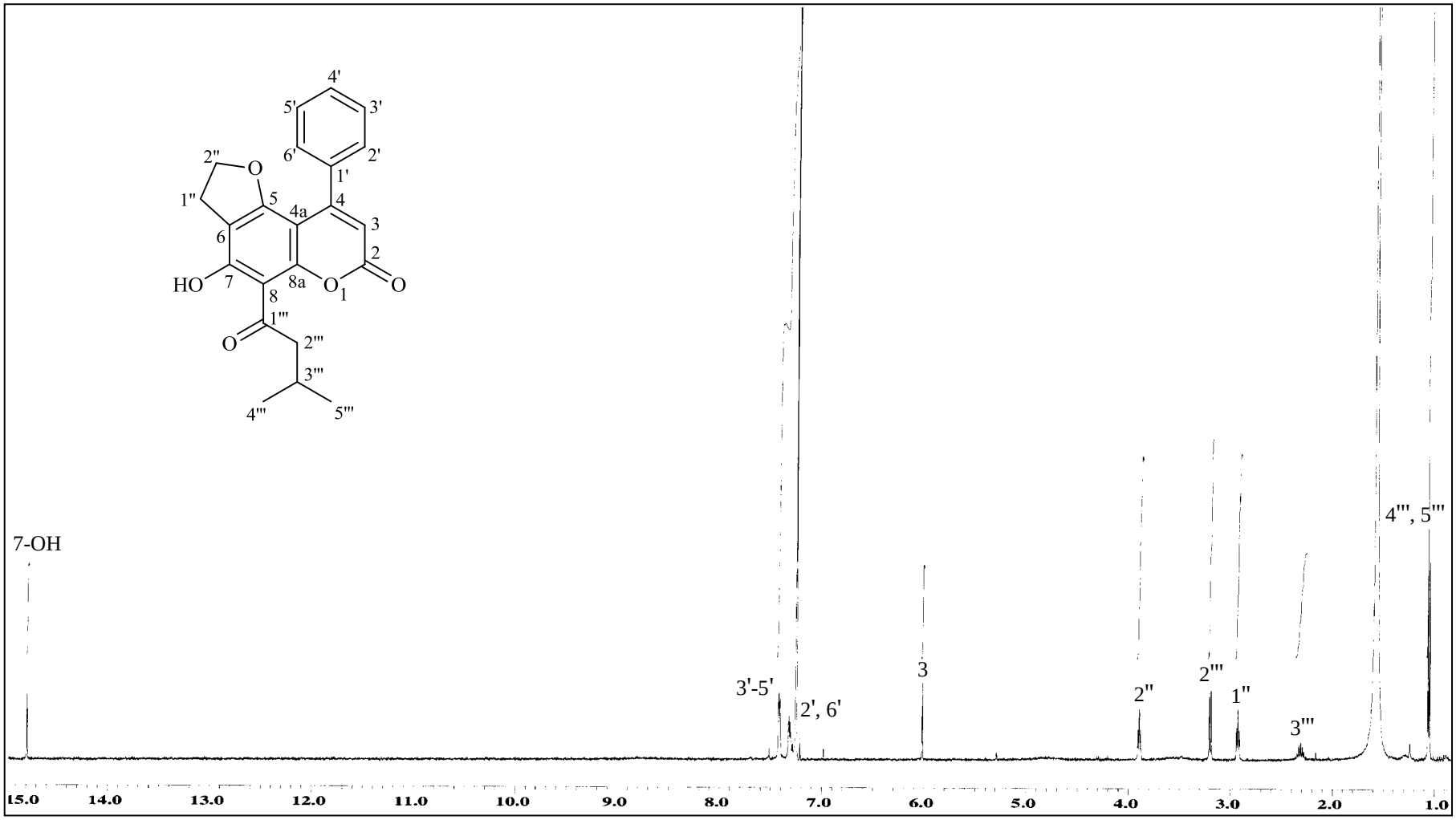


Figure 3.38: ^{1}H NMR Spectrum of Compound R **199**

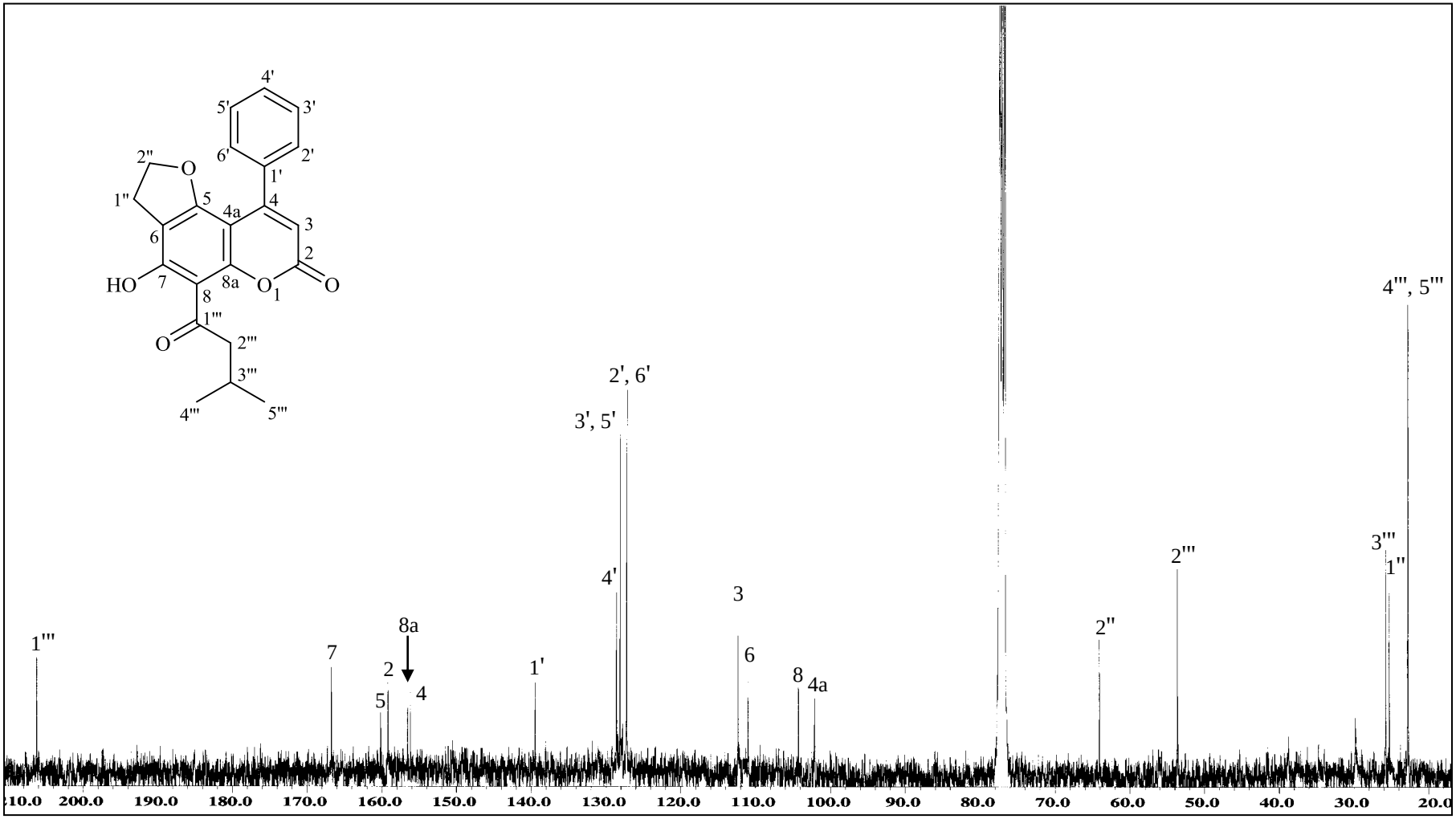
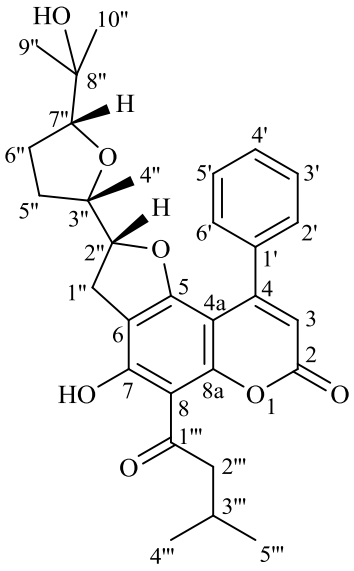


Figure 3.39: ^{13}C NMR Spectrum of Compound R **199**

3.1.19 Compound S: Mesuagenin I 200



200

Separation using column chromatography, followed by HPLC afforded compound S as a white amorphous solid. The HRESIMS measurement of its $[M+Na]^+$ ion at *m/z* 529.2209 (calculated 529.2202) suggested a molecular formula of $C_{30}H_{34}O_7$. The UV spectrum (EtOH) supported an 8-acyl-5,7-dioxycoumarin type, with absorptions at λ_{max} 233, 287 and 333 nm[82]. The IR spectrum showed absorptions at υ_{max} 3415 (free hydroxyl), 1740 (α,β-unsaturated lactone), 1603 (chelated acyl) and 1385 cm^{-1} (geminal-dimethyl)[47, 85].

The ^{13}C NMR spectrum of compound S showed thirty carbon resonances; five methyls, four methylenes, nine methines and twelve quaternary carbons. In the ^1H NMR spectrum of compound S, the typical H-3 singlet of a 4-substituted coumarin was observed at δ 6.03. This characteristic singlet of H-3 was further corroborated with the presence of two sets of multiplets, centered at δ 7.39 and 7.27, corresponding to three (H-3'-H5') and two aromatic (H-2', H-6') protons, respectively. The ^1H NMR spectrum of compound S also displayed the presence of a chelated hydroxyl group at δ 14.29,

which was exchangeable with D_2O. All the observations supported the presence of an 8-acyl-5,7-dioxy-4-phenylcoumarin type skeleton, which was also indicated by the UV data.

Compound S showed similar NMR profiles with those of compound O. However, C-3" of compound S appeared to be more downfield at δ 83.1 as compared to compound O, which was at δ 73.4. In addition, C-7" also appeared as a sp^3 methine at δ 85.5 in compound S, as compared to an sp^2 methine at δ 123.9 in compound O. An oxymethine was also in evidence in the ^{13}C NMR spectrum of compound S at δ 71.0 (C-8"). These observations indicated that a hydroxylated, cyclized prenyl ring was attached to C-2" of the furan moiety in compound S. The connectivity of the prenyl ring in the molecule was further supported by the following correlations in the HMBC spectrum of compound S, H-5"/C-2", 3", 4", 6" and 7".

In the NOESY spectrum of compound S, cross correlation was observed between H-2" and H-4", which suggested that these two protons were located in the same plane. Additionally, H-7" was also correlated with H-4", which placed H-2", H-4" and H-7" in the same plane.

A careful search through SciFinder database led to the result that compound S was indeed a new chemical compound, which was named as mesuagenin I **200**.

Table 3.20: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound S **200**

Position	δ_H, *J* (Hz)	δ_C	NOESY	HMBC
2	-	159.2		
3	6.03 (1H, *s*)	111.3		2, 4a, 1'
4	-	155.1		
4a	-	98.6		
5	-	162.6		
6	-	109.9		
7-OH	14.29 (1H, *s*)	163.7		6, 7, 8
8	-	105.0		
8a	-	157.6		
1'	-	137.8		
2'	7.27 (1H, *m*, Ar)	127.7		
3'	7.39 (3H, *m*, Ar)	127.9		1'
4'		128.8		2', 6'
5'		127.9		1'
6'	7.27 (1H, *m*, Ar)	127.7		
1"	3.07 (2H, *d*, *J* = 9.2)	26.8	2", 4", 5"	5, 6, 2", 3"
2"	4.57 (1H, *t*, *J* = 9.2)	89.2	1", 4", 5", 6"	5, 4", 5"
3"	-	83.1		
4"	0.97 (3H, *s*)	20.6		2", 3", 5"
5"	1.43 (2H, *dd*, *J* = 6.1, 8.6)	35.1		2", 3", 4", 6", 7"
6"	1.74 (2H, *m*)	25.9		3", 7", 8"
7"	3.71 (1H, *dd*, *J* = 6.1, 8.6)	85.5	4", 5", 6", 9", 10"	
8"	-	71.0		
9"	1.03 (3H, *s*)	24.4		7", 8", 10"
10"	1.12 (3H, *s*)	27.4		7", 8", 9"
1'"	-	206.1		
2'"	3.16 (2H, *d*, *J* = 6.7)	53.5		1'", 3'", 4'", 5'"
3'"	2.31 (1H, *m*)	25.7		1'", 2'", 4'", 5'"
4'"	1.05 (6H, *d*, *J* = 6.7)	22.8		3'", 5'"
5'"		22.8		3'", 4'"

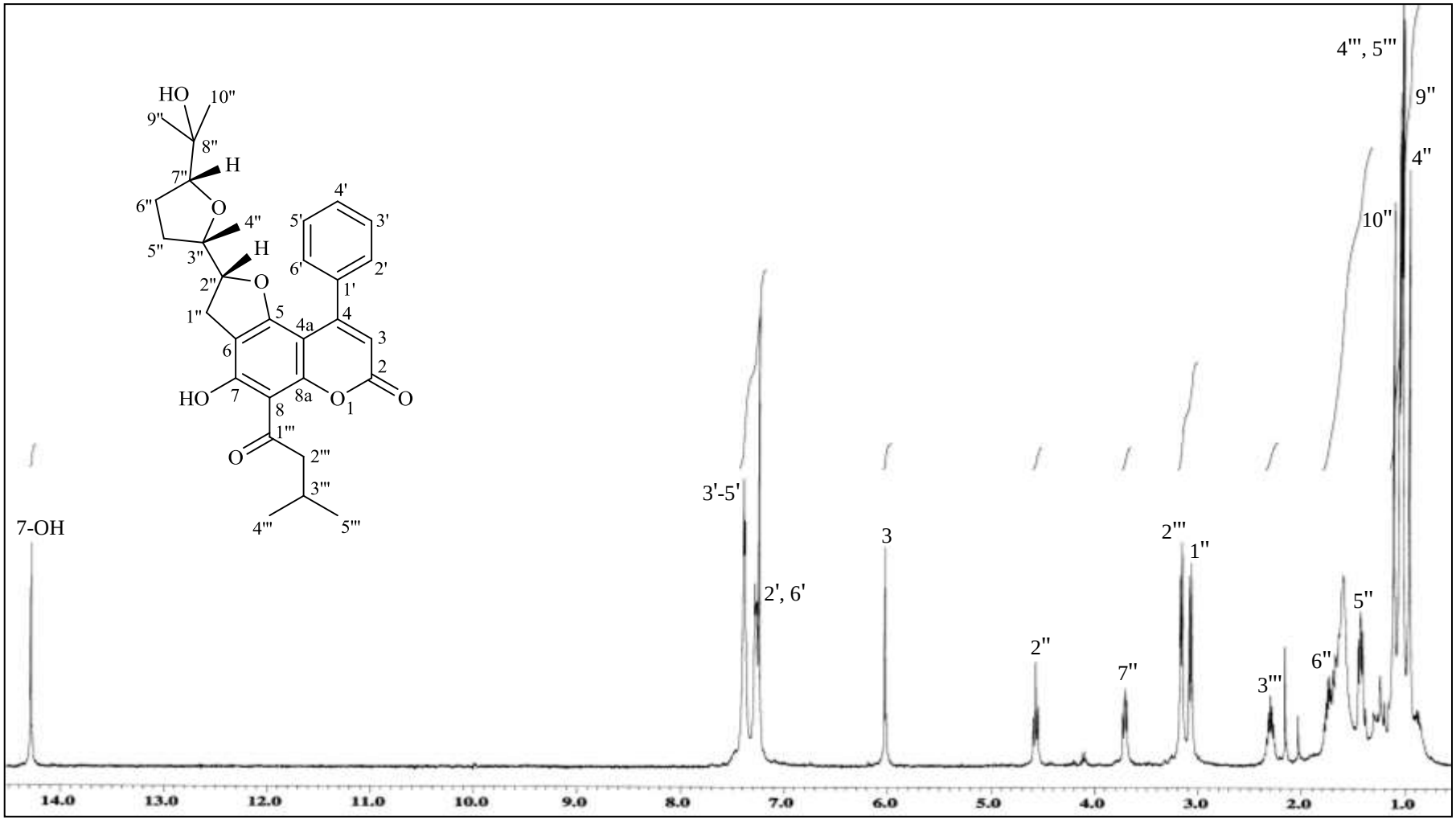
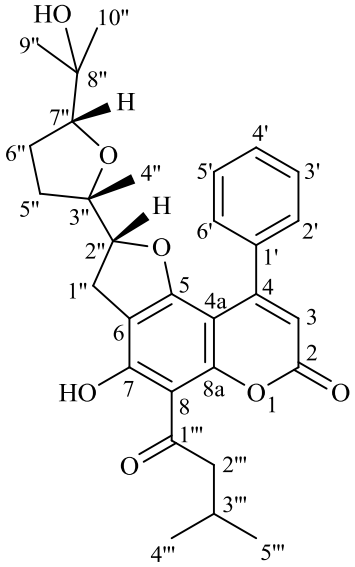


Figure 3.40: ^{1}H NMR Spectrum of Compound S **200**



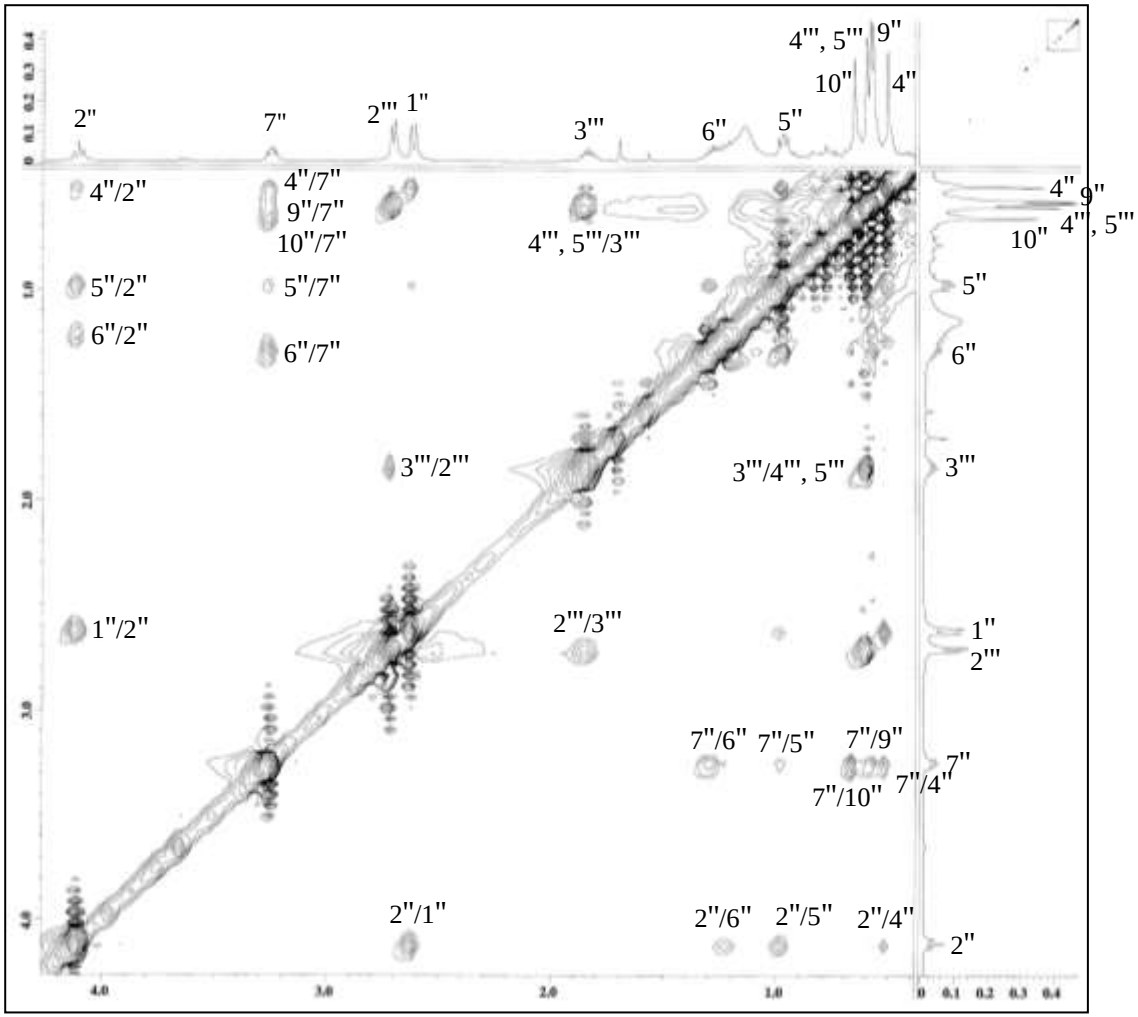
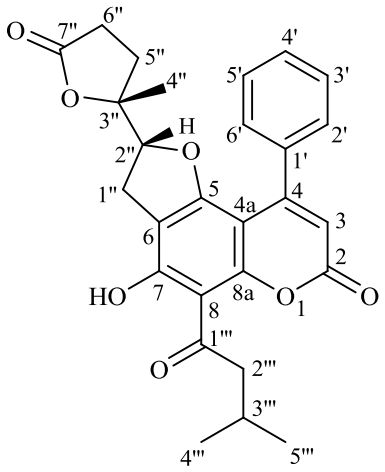


Figure 3.41: NOESY Spectrum of Compound S **200**

3.1.20 Compound T: Mesuagenin J 201



201

Compound T was obtained as a yellowish oil and has a molecular formula of $C_{27}H_{26}O_7$ based on the HRESIMS measurement; $[M+Na]^+$ ion at *m/z* 485.1577 (calculated 485.1576). The IR spectrum accumulated using a neat film technique showed evidence of strong absorptions at υ_{max} 3445 (OH group), 1743 (α-pyrone), 1603 (chelated acyl group) and 1384 cm^{-1} (geminal dimethyl group)[47, 88]. Interestingly, the IR spectrum accumulated using the ATR technique showed strong absorption at υ_{max} 1770 cm^{-1}, which indicated the presence of a five-membered dihydrofuran-2(3*H*)-one functionality[95]. The UV spectrum (EtOH) of compound T could readily be associated with an 8-acyl-5,7-dioxycoumarin type, due to absorptions at λ_{max} 230, 285 and 339 nm[82].

A total of twenty-seven carbon signals were observed in the ^{13}C NMR spectrum of compound T; three methyls, four methylenes, eight methines and twelve quaternary carbons. The number of carbon signals for compound T was considered abnormal compared to the usual thirty-carbon compounds from this study. However, the spectral data of compound T showed similarities with compound O, namely a typical H-3 signal

of a 4-phenylcoumarin (δ 6.05, *s*, H-3), a monosubstituted phenyl (δ 7.41 and 7.27, 5H, *m*, H-2'-H6'), a chelated hydroxyl group (δ 14.30, *s*, 7-OH) and a 3-methylbutanoyl moeity [δ 3.17 (2H, *d*, *J* = 6.8 Hz, H-2'''), 2.30 (1H, *m*, H-3''') and 1.05 (6H, *d*, *J* = 6.8 Hz, H-4''' and H-5''')].

The differences in the coupling pattern of the remaining substituents of C-5/C-6 were noticeable by the absence of the methyls C-9'' and C-10'' in the ^{1}H and ^{13}C NMR spectra of compound T as compared to compound O. Moreover, split coupling patterns were visible for methylenes of C-5'' and C-6''; δ 1.75 (1H, *m*, H-5''α), 1.85 (1H, *m*, H-5'' β), 2.28 (1H, *m*, H-6''α) and 2.45 (1H, *m*, H-6''β). A carbonyl peak was also visible in the ^{13}C NMR spectrum of compound T at δ 175.4 (C-7''). These observations indicated the presence of a five-membered dihydrofuran-2(3*H*)-one ring in the molecule, which was supported by the IR spectrum using the ATR technique earlier[95].

The furan ring resonances connected to C-5/C-6 were visible in the ^{1}H NMR spectrum of compound T; a set of double doublets at δ 2.89 (1H, *dd*, *J* = 8.8, 16.1 Hz, H-1''α) and 3.19 (1H, *dd*, *J* = 8.8, 16.1 Hz, H-1''β), and another double doublet at δ 4.74 (1H, *dd*, *J* = 8.8, 10.2 Hz, H-2''). The connectivity between the five-membered dihydrofuran-2(3*H*)-one ring (C-3'' - C-7'') and the furan ring of C-5/C-6 was evident in the HMBC spectrum of compound T, H-1''α,β/C-5, C-6, C-2'' and C-3''.

In the NOESY spectrum of compound T, cross correlation was observed between H-2'' and H-4'', which suggested that these two protons were located in the same plane.

The observed spectral data of compound T and a thorough search in the SciFinder database revealed that this compound was a new chemical entity, which was named as mesuagenin J **201**.

Table 3.21: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound T **201**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	158.9		
3	6.05 (1H, *s*)	111.6		2, 4a, 1'
4	-	155.0		
4a	-	98.9		
5	-	161.7		
6	-	109.1		
7-OH	14.30 (1H, *s*)	163.6		6, 7, 8, 1'''
8	-	105.4		
8a	-	157.7		
1'	-	137.5		
2'	7.27 (1H, *m*, Ar)	127.4		4, 4'
3'	7.41 (3H, *m*, Ar)	128.0		1'
4'		129.3		2', 6'
5'		128.0		1'
6'	7.27 (1H, *m*, Ar)	127.4		4, 4'
1"α	2.89 (1H, *dd*, *J* = 8.8, 16.1)	27.2	2", 1"β	5, 6, 2", 3"
1"β	3.19 (1H, *dd*, *J* = 8.8, 16.1)		2", 1"α	5, 6, 2", 3"
2"	4.74 (1H, *dd*, *J* = 8.8, 10.2)	89.1	1"α, 1"β	5"
3"	-	85.4		
4"	1.19 (3H, *s*)	22.3		2", 3", 5"
5"α	1.75 (1H, *m*)	28.9	5"β, 6"α, 6"β	2", 3", 4", 7"
5"β	1.85 (1H, *m*)		5"α, 6"α, 6"β	2", 3", 4", 7"
6"α	2.28 (1H, *m*)	28.3	5"α, 5"β, 6"β	3", 7"
6"β	2.45 (1H, *m*)		5"α, 5"β, 6"α	3", 7"
7"	-	175.4		
1'"	-	206.3		
2'"	3.17 (2H, *d*, *J* = 6.8)	53.6	3'"	1'", 3'", 4'", 5'"
3'"	2.30 (1H, *m*)	25.7	2'", 4'", 5'"	2'", 4'", 5'"
4'"	1.05 (6H, *d*, *J* = 6.8)	22.8	3'", 5'"	2'", 3'", 5'"
5'"		22.8	3'", 5'"	2'", 3'", 4'"

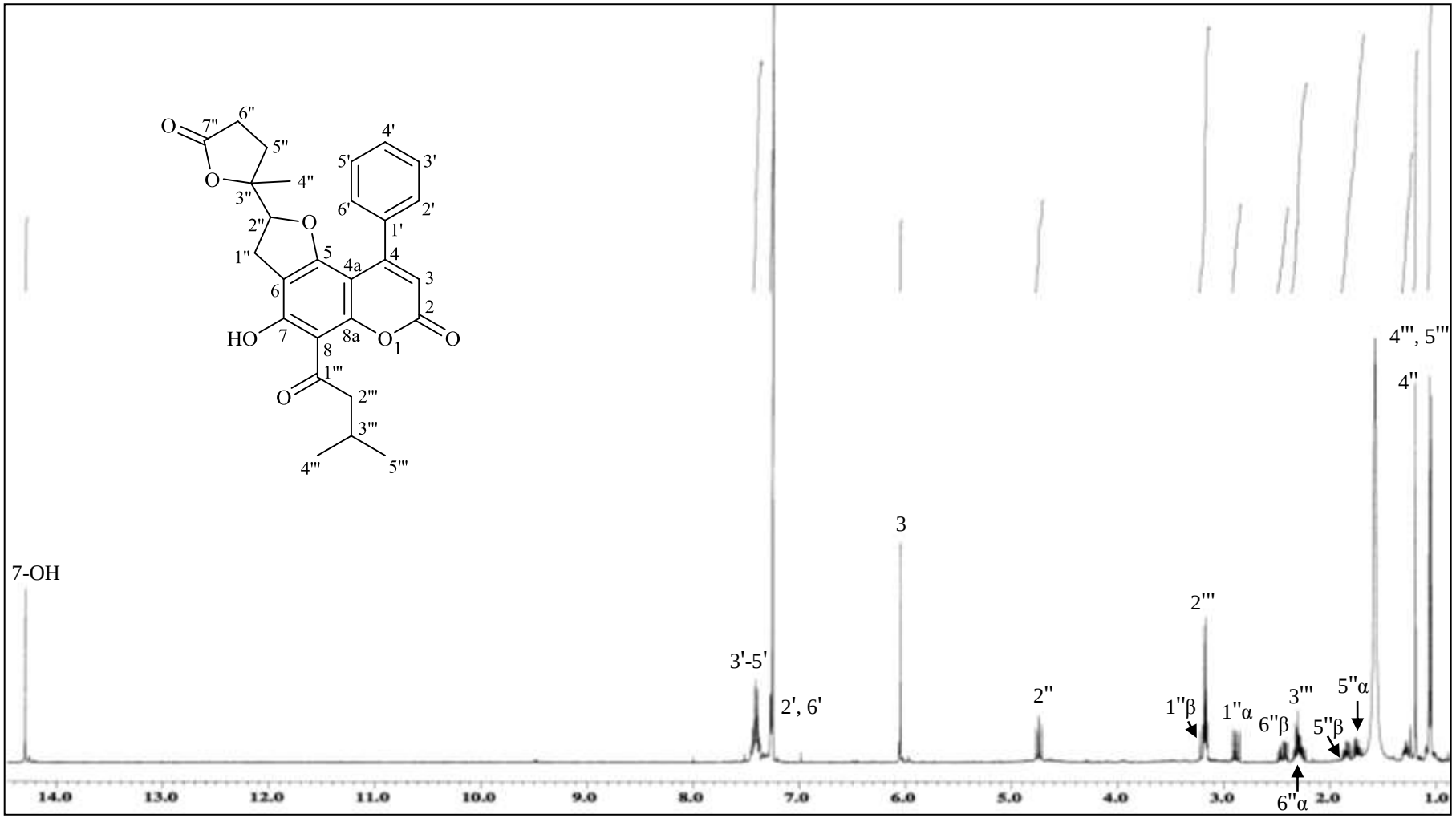


Figure 3.42: ^{1}H NMR Spectrum of Compound T **201**

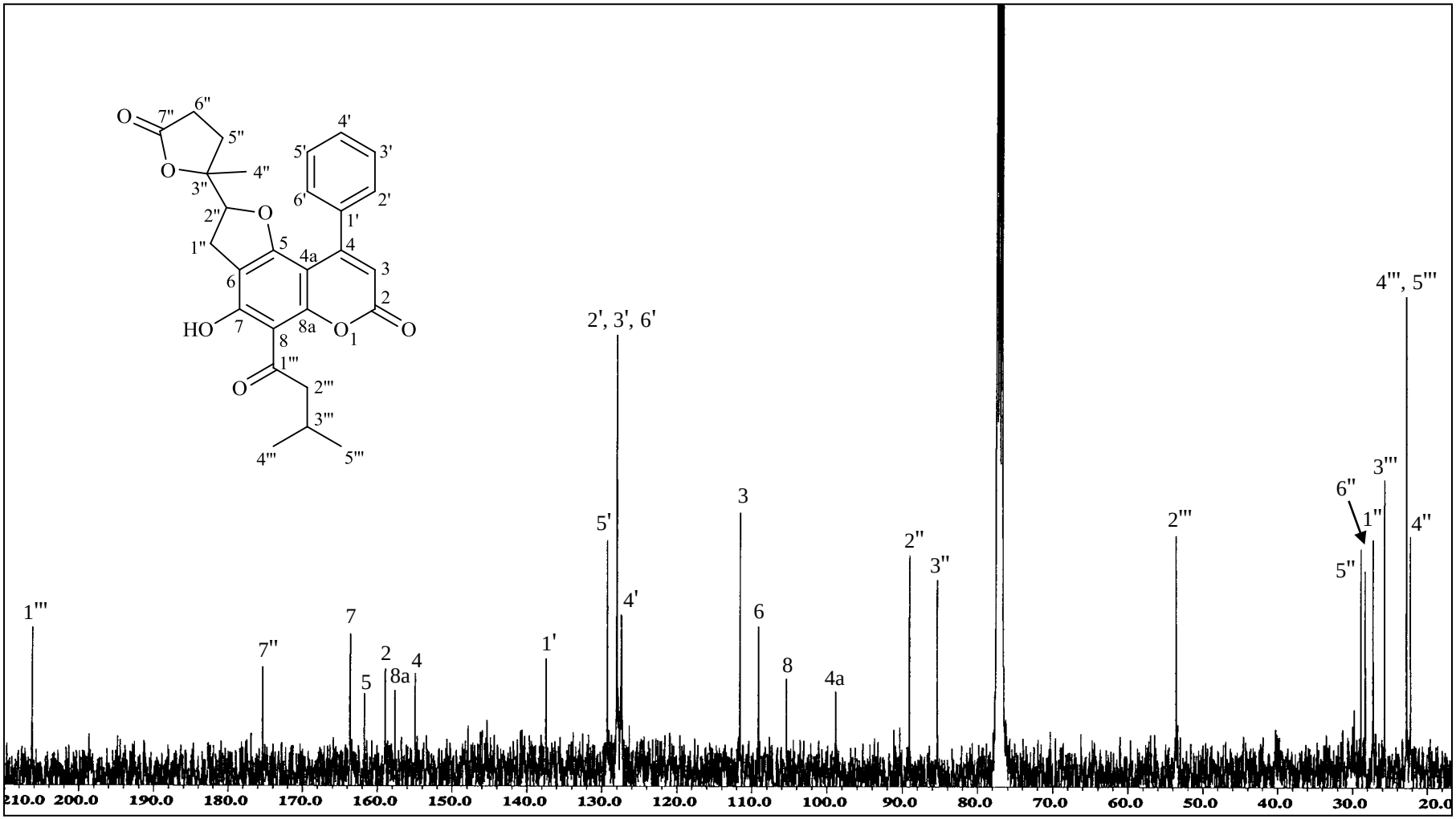
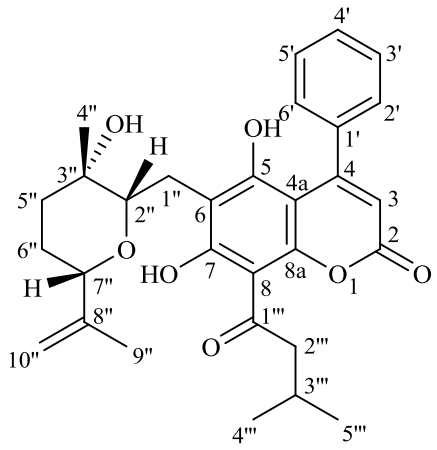


Figure 3.43: ^{13}C NMR Spectrum of Compound T **201**

3.1.21 Compound U: Mesuagenin K 202



202

Compound U was isolated as a yellowish oil using column chromatography fractionation, followed by HPLC isolation. The HRESIMS spectrum revealed a pseudomolecular ion of $[M+Na]^+$ at *m/z* 529.2142 (calculated 529.2202), which corresponded to the molecular formula of $C_{30}H_{34}O_7$. The UV spectrum (EtOH) supported an 8-acyl-5,7-dihydroxycoumarin type, with absorptions at λ_{max} 237, 284 and 335 nm[82].The IR spectrum showed absorptions at υ_{max} 1388 cm^{-1} belonging to geminal dimethyl and 1597 cm^{-1} which belongs to chelated acyl group. The IR spectrum also exhibited a strong peak at 1738 cm^{-1} due to the presence of an α-pyrone, and a broad peak at 3356 cm^{-1} due to OH stretching[47, 88].

The ^{13}C NMR spectrum showed the presence of four methyls, five methylenes, nine methines and twelve quaternary signals. In the ^1H NMR spectrum, the typical H-3 singlet of 4-phenylcoumarin moiety was observed at δ 6.03. The presence of a mono-substituted phenyl group at C-4 of a 4-phenylcoumarin was corroborated by the presence two sets of multiplets, centred at δ 7.29 and δ 7.39, corresponding to two (H-2' and H-6') and three (H-3', H-4' and H-5') aromatic protons, respectively. The ^1H NMR

spectrum also displayed the presence of hydroxyl group peaks revealing a broad singlet at δ 9.51 (5-OH) and a sharp singlet peak at δ 14.82 (7-OH), in which both were exchangeable with D_2O. In addition, the broad OH absorption depicted from the IR spectrum further supported this observation. These data suggested the presence of an 8-acyl-5,7-dihydroxy-4-phenylcoumarin type.

In addition, the ^{1}H NMR spectrum of compound U showed a set of double doublets at δ 2.42 (1H, *dd*, *J* = 9.4, 15.4 Hz, H-1"α) and 3.17 (1H, *dd*, *J* = -, 15.4 Hz, H-1"β), and a doublet at δ 3.75 (1H, *d*, *J* = 9.4 Hz, H-2"). These peaks, along with the presence, of the 5-OH broad singlet at δ 9.51, were devoid from the normal cyclised furan ring system as present in compound J. The connectivity of C-1" with the coumarin skeleton at position C-6 could be deduced by the following correlations in the HMBC spectrum, H-1"α,β/C-5, C-6, C-7 and C-2".

A methyl singlet at δ 1.31 (H-4") and a broad singlet of a hydroxyl at δ 3.39 (3"-OH) were also apparent in the ^{1}H NMR spectrum of compound U. A pyran system can also be inferred by these peaks displayed in the ^{1}H NMR spectrum; δ 1.62 (1H, *m*, H-5"α), 1.84 (1H, *m*, H-6"α), 2.04 (1H, *m*, H-6"β), 2.05 (1H, *m*, H-5"β) and 4.25 (1H, *dd*, *J* = 5.4, 10.7 Hz, H-7"). In the HMBC spectrum of compound U, the connection between H-2" and H-5" in the cyclic system were visible when H-2" showed correlations with C-3" (δ 85.8) and C-5" (δ 30.6).

Furthermore, a propene unit was also visible in the ^{1}H NMR spectrum as a methyl singlet at δ 1.70 (H-9") and two singlets of a methylene group at δ 4.81 (H-10"α) and 4.98 (H-10"β). All these peaks gave cross correlations with each other, and also with C-7" (δ 84.4) in the HMBC spectrum, thus establishing the connectivity between the pyran

system and the propene unit. All these observations led to the identification of a 3-hydroxy-3-methyl-6-(prop-1-en-2-yl)tetrahydro-2*H*-pyran-2-yl)methyl substituent to be present in the molecule.

The COSY spectrum revealed cross correlations between δ 3.24 (2H, *dd*, *J* = 5.5, 6.7 Hz, H-2'''), δ 2.33 (1H, *m*, H-3''') and δ 1.06 (6H, *d*, *J* = 6.7 Hz, H-4''' and H-5'''). In the HMBC spectrum of compound U, the methylene protons (H-2''') and the methine proton (H-3''') gave cross-peaks with a keto function at δ 206.3, belonging to C-1'''. With this, it was identified that a 3-methylbutanoyl chain was the other substituent of the 4-phenylcoumarin, which was attached at C-8.

Cross correlations between the protons H-2''/H-4'' and H-7'' were observed in the NOESY spectrum of compound U, which suggested that these two protons were in the same plane. Based on this information, the relative stereochemistry of the carbon centers C-2'', C-3'' and C-7'' was assigned.

In view of the above data, and with a thorough search in the SciFinder database, it was deduced that compound U is a new chemical entity with the proposed structure, which was named as mesuagenin K **202**.

Table 3.22: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 600 MHz) of Compound U **202**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	159.2		
3	6.03 (1H, *s*)	112.3		2, 4a, 1'
4	-	156.6		
4a	-	102.5		
5-OH	9.51 (1H, brs)	160.6		4a
6	-	110.3		
7-OH	14.82 (1H, *s*)	166.6		6, 7, 8, 1'''
8	-	104.2		
8a	-	156.7		
1'	-	140.1		
2'	7.29 (1H, *m*, Ar)	127.2		
3'	7.39 (3H, *m*, Ar)	127.7		1'
4'		128.1		2', 6'
5'		127.7		1'
6'	7.29 (1H, *m*, Ar)	127.2		
1"α	2.42 (1H, *dd*, *J* = 9.4, 15.4)	25.2	2"	5, 6, 7, 2"
1"β	3.17 (1H, *dd*, *J* = - , 15.4)		2"	5, 6, 7, 2", 3"
2"	3.75 (1H, *d*, *J* = 9.4)	78.6	1"α, β, 4", 5"α	6, 3", 4", 5"
3"-OH	3.39 (1H, brs)	85.8		
4"	1.31 (3H, *s*)	24.1	1"α, β, 2", 5"α, 6"α, 10"β	2", 3", 5"
5"α	1.62 (1H, *m*)	30.6		4", 7"
5"β	2.05 (1H, *m*)			2", 3", 4", 6"
6"α	1.84 (1H, *m*)	31.4		5", 7", 8"
6"β	2.04 (1H, *m*)			
7"	4.25 (1H, *dd*, *J* = 5.4, 10.7)	84.4	5"α, 6"α, 9", 10"β	6", 10"
8"	-	145.3		
9"	1.70 (3H, *s*)	17.8	7", 10"α, β	6", 7", 8", 10"
10"α	4.81 (1H, *s*)	110.9	7", 9"	7", 8", 9"
10"β	4.98 (1H, *s*)		7", 9"	7", 8", 9"
1'''	-	206.3		
2'''	3.24 (2H, *dd*, *J* = 5.5, 6.7)	53.6	3''', 4''', 5'''	1''', 3''', 4''', 5'''
3'''	2.33 (1H, *m*)	25.8	2''', 4''', 5'''	1''', 2''', 4''', 5'''
4'''	1.06 (6H, *d*, *J* = 6.7)	22.8	2''', 3'''	3''', 5'''
5'''		22.8	2''', 3'''	3''', 4'''

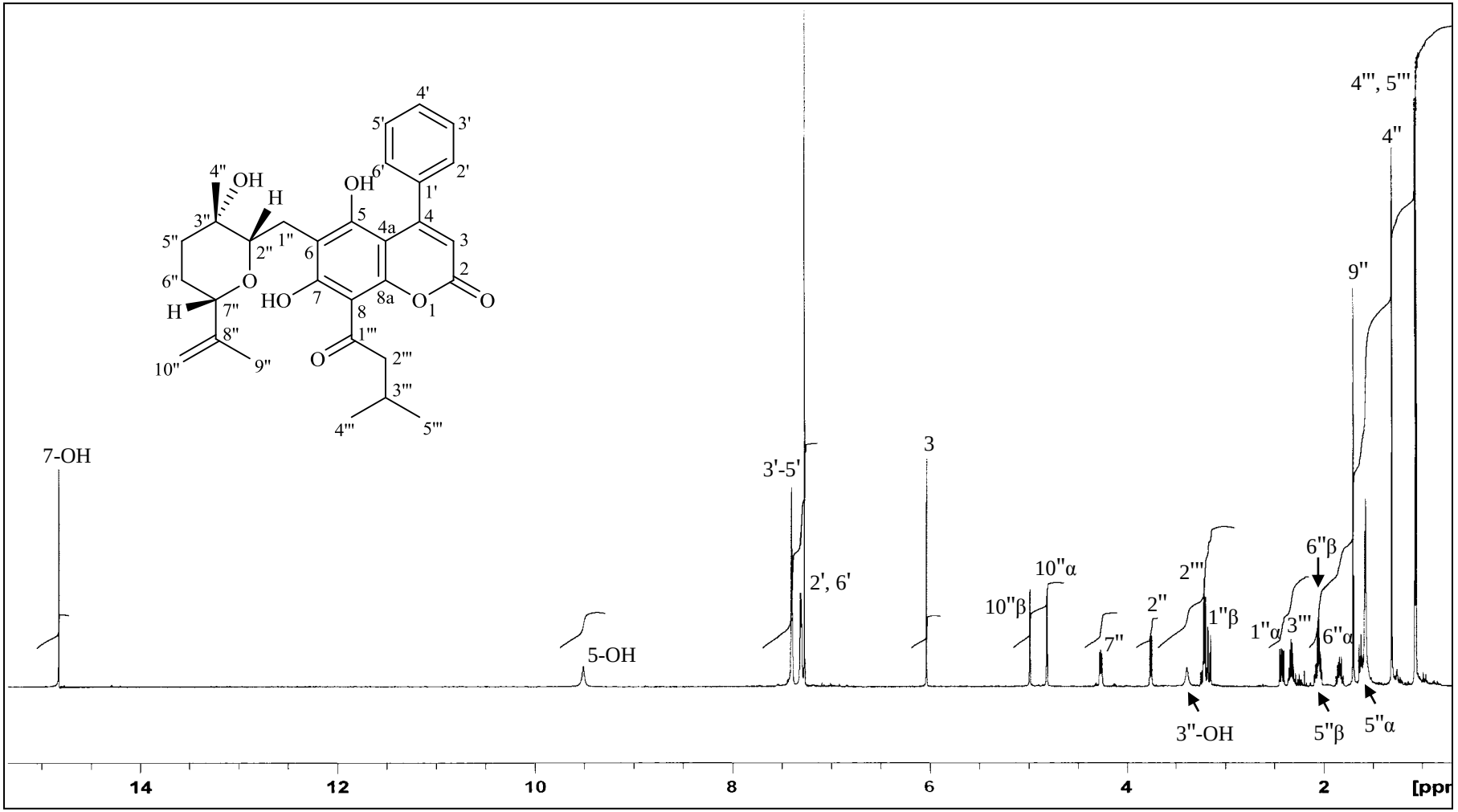


Figure 3.44: ^{1}H NMR Spectrum of Compound U **202**

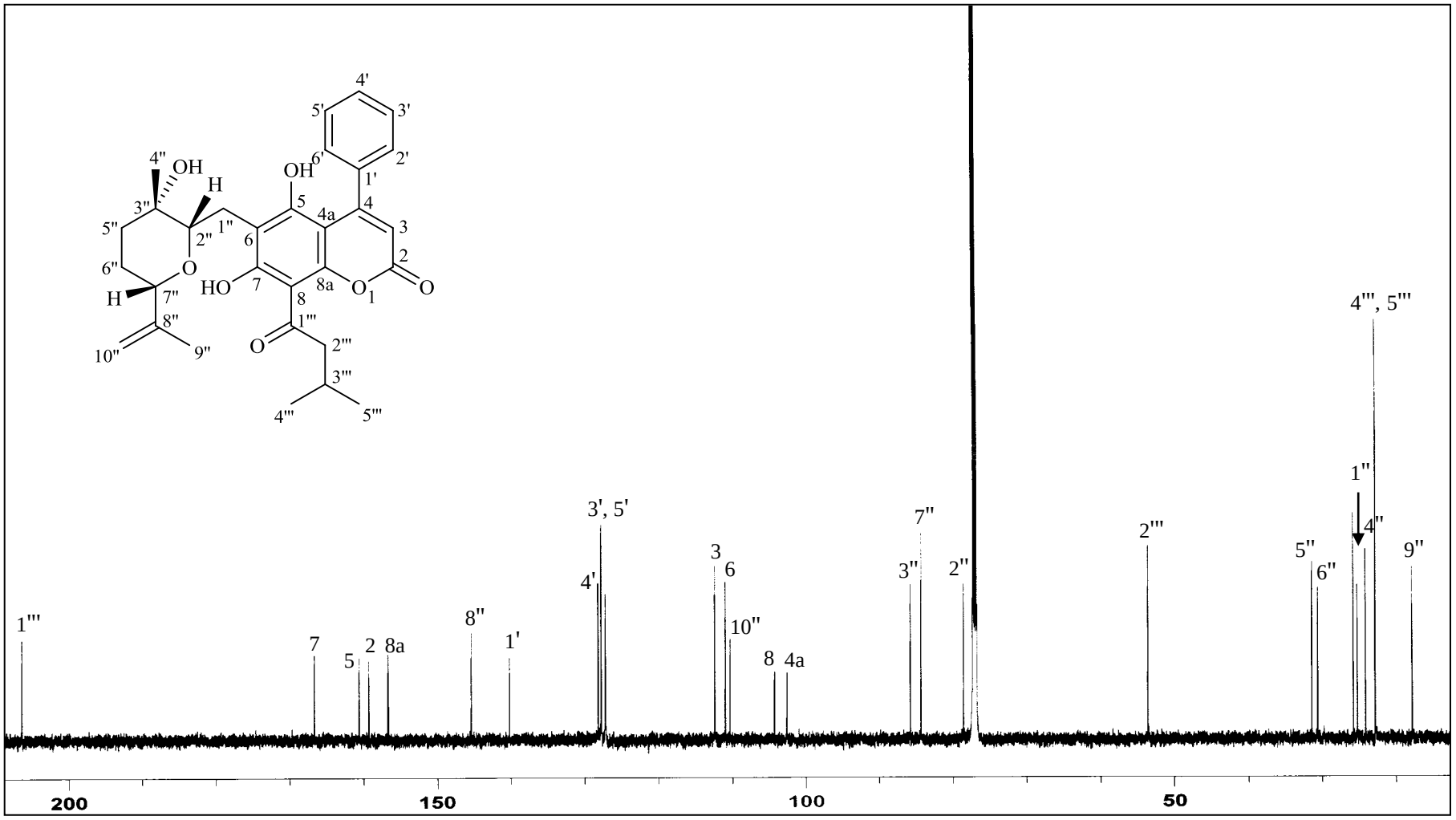
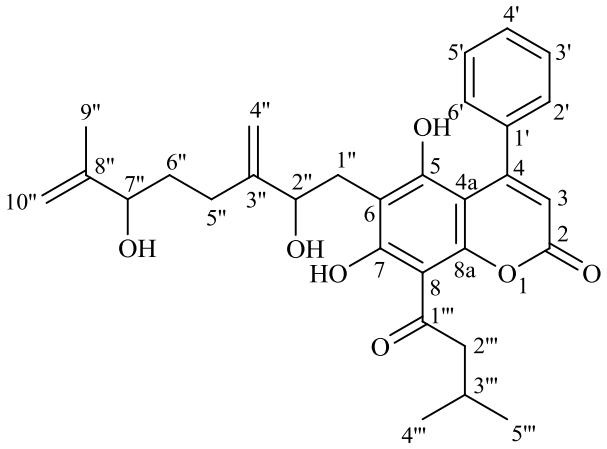


Figure 3.45: ^{13}C NMR Spectrum of Compound U **202**

3.1.22 Compound V: Mesuagenin L 203



203

Compound V was separated as a white amorphous solid. The molecular formula of compound V was determined by the HRESIMS measurement of its $[M+Na]^+$ ion at *m/z* 529.2145 (calculated 529.2202), as $C_{30}H_{34}O_7$. The UV spectrum (EtOH) supported an 8-acyl-5,7-dioxycoumarin type, with absorptions at λ_{max} 233, 285 and 337 nm[82]. The IR spectrum showed absorptions at υ_{max} 3451 (free OH), 1743 (α,β-unsaturated lactone), 1603 (chelated acyl group) and 1385 cm^{-1} (geminal dimethyl group)[47, 85].

The ^{13}C NMR spectrum of compound V displayed a total of thirty carbons; three methyls, six methylenes, nine methines and twelve quaternary carbons. The ^1H NMR spectrum of compound V showed striking similarities with the ^1H NMR spectrum of compound J, with similar substituents observed at position C-4 (monosubstituted phenyl; δ 7.31 and 7.40, 5H, *m*, H-2'-H6'), C-7 (chelated hydroxyl group; δ 14.80, *s*, 7-OH) and C-8 [3-methylbutanoyl; δ 3.20 (2H, *t*, *J* = 6.7 Hz, H-2'''), δ 2.32 (1H, *m*, H-3''') and δ 1.06 (6H, *d*, *J* = 6.7 Hz, H-4''' and H-5''')].

However, examination of the ^1H NMR spectrum of compound V showed an obvious difference in the coupling pattern of the substituent group at position C-6. The singlet of

H-4" methyl at δ 1.31 in the ^{1}H NMR spectrum of compound J was missing in the ^{1}H NMR spectrum of compound V. On the other hand, two singlets of an sp^2 methylene, integrating for one proton each, were visible in the ^{1}H NMR spectrum of compound V; δ 4.92 (1H, *s*, H-4"α) and δ 5.07 (1H, *s*, H-4"β). Furthermore, in the HMBC spectrum of compound V, both the H-4"α and H-4"β resonances correlated with δ 76.6 (C-2") and δ 28.3 (C-5"). Therefore, it was deduced that a double bond was present between C-3" (δ 150.6) and C-4" (δ 110.3).

The methyl of C-10" was also missing in the ^{1}H NMR spectrum of compound V as compared to compound J. However, this methyl was replaced by an sp^2 methylene; δ 4.87 (1H, *s*, H-10"α) and δ 4.95 (1H, *s*, H-10"β). The sp^2 methylene protons of C-10" correlated with δ 76.0 (C-7") and δ 17.8 (C-9") in the HMBC spectrum, thus confirming its location.

After a thorough search in the SciFinder database, it was found that compound V was a new chemical entity, which was named as mesuagenin L **203**.

Table 3.23: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 600 MHz) of Compound V **203**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	159.2		
3	6.03 (1H, *s*)	112.2		2, 4a, 1'
4	-	156.3		
4a	-	102.2		
5-OH	9.40 (1H, br*s*)	160.5		
6	-	110.0		
7-OH	14.80 (1H, *s*)	166.8		6, 7, 8
8	-	104.1		
8a	-	156.6		
1'	-	139.8		
2'	7.31 (1H, *m*, Ar)	127.1		4, 4'
3'	7.40 (3H, *m*, Ar)	127.8		1'
4'		128.2		2', 6'
5'		127.8		1'
6'	7.31 (1H, *m*, Ar)	127.1		4, 4'
1"α	2.72 (2H, *dd*, *J* = 8.5, 14.9)	29.3	2", 1"β	5, 6, 7, 2"
1"β	3.12 (2H, *dd*, *J* = - , 14.9)		2", 1"α	5, 6, 7, 3"
2"	4.33 (1H, *d*, *J* = 8.5)	76.6	1"α, β	6, 3", 5"
3"	-	150.6		
4"α	4.92 (1H, *s*)	110.3		2", 5"
4"β	5.07 (1H, *s*)			2", 3", 5"
5"α	2.17 (1H, *m*)	28.3	5"β, 6"α, β	3", 4", 6"
5"β	2.27 (1H, *m*)		5"α, 6"α, β	2", 3", 4", 6"
6"α	1.68 (1H, *m*)	33.1	5"α, β, 6"β	5", 7"
6"β	1.80 (1H, *m*)		5"α, β, 6"α	
7"	4.10 (1H, *dd*, *J* = 4.8, 7.6)	76.0	9", 6"α, β	6", 10"
8"	-	147.1		
9"	1.73 (3H, *s*)	17.8	7", 10"α, β	7", 8", 10"
10"α	4.87 (1H, *s*)	111.4	9"	7", 9"
10"β	4.95 (1H, *s*)		9"	7", 8", 9"
1'"	-	206.1		
2'"	3.20 (2H, *t*, *J* = 6.7)	53.5	3'", 4'", 5'"	1'", 3'", 4'", 5'"
3'"	2.32 (1H, *m*)	25.7	2'", 4'", 5'"	2'", 4'", 5'"
4'"	1.06 (6H, *d*, *J* = 6.7)	22.7	2'", 3'"	2'", 3'", 5'"
5'"		22.7	2'", 3'"	2'", 3'", 4'"

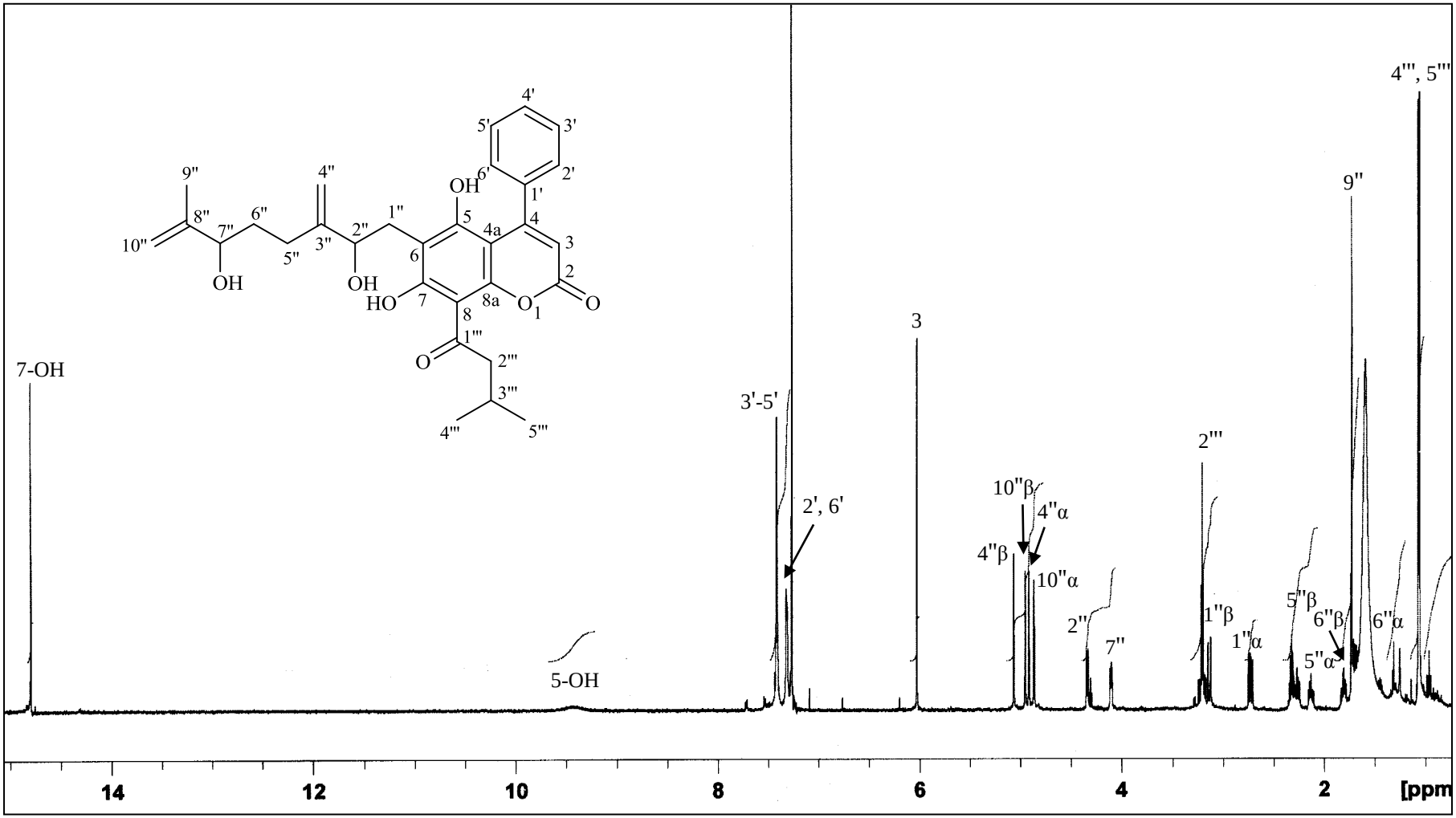
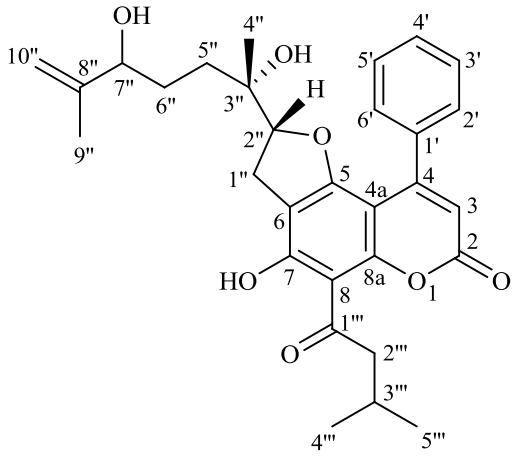


Figure 3.46: ^{1}H NMR Spectrum of Compound V **203**

3.1.23 Compound W: Mesuagenin M 204



204

Compound W was obtained as a white amorphous solid using column chromatography fractionation, followed by HPLC separation. The HRESIMS measurement of its $[M+Na]^+$ ion at *m/z* 529.2146 (calculated 529.2202) suggested a molecular formula of $C_{30}H_{34}O_7$. The UV spectrum (EtOH) supported an 8-acyl-5,7-dioxycoumarin type, with absorptions at λ_{max} 233, 285 and 337 nm[82]. The IR spectrum showed absorptions at υ_{max} 1740 (α,β-unsaturated lactone) and 1603 cm^{-1} (chelated acyl group)[47, 85].

Compound W showed similar profiles with those of compound O. Interestingly, compound W has an extra 16 molecular mass unit with respect to compound O. This suggested the presence of an additional hydroxyl group in compound W. An inspection of the ^1H and ^{13}C NMR spectra of compound W and compound O led to the following observations. Firstly, the existence of an extra oxymethine carbon and proton at δ 75.7 (C-7") and 4.02 (1H, br*t*, *J* = 6.6 Hz, H-7"), respectively. Secondly, the presence of a terminal sp^2 carbon at δ 111.2 (C-10") in compound W. These observations indicated that the sp^2 C-7" methine in compound O has been hydroxylated to an sp^3 carbon in compound W. In addition, the methyl C-10" in compound O was dehydrogenated into a methylene carbon in compound W.

In the NOESY spectrum of compound W, a cross correlation was observed between H-2" and H-4", suggesting that these two protons were situated in the same plane.

A careful search through the SciFinder database led to the result that compound W was a new chemical compound, which was named as mesuagenin M **204**.

Table 3.24: ^{1}H NMR, ^{13}C NMR, NOESY and HMBC (in $CDCl_3$, 600 MHz) of Compound W **204**

Position	δ_H, *J* (Hz)	δ_C	NOESY	HMBC
2	-	159.1		
3	6.06 (1H, *s*)	111.1	2', 6'	2, 4a, 1'
4	-	154.9		
4a	-	98.6		
5	-	161.9		
6	-	110.1		
7-OH	14.32 (1H, *s*)	163.7		6, 7, 8, 1'''
8	-	105.0		
8a	-	157.4		
1'	-	138.1		
2'	7.30 (1H, *m*, Ar)	127.5	3	
3'	7.42 (3H, *m*, Ar)	127.9		1'
4'		128.7		2', 6'
5'		127.9		1'
6'	7.30 (1H, *m*, Ar)	127.5	3	
1"α	3.02 (1H, *dd*, *J* = 9.8, 15.4)	26.4	2"	5, 6, 2", 3"
1"β	3.06 (1H, *dd*, *J* = 9.8, 15.4)		2"	5, 6, 1"
2"	4.56 (1H, *t*, *J* = 9.8)	91.9	1"α, 1"β, 4"	1", 4", 5"
3"-OH	-	72.8		
4"	0.86 (3H, *s*)	21.7	2"	2", 3", 5"
5"	1.30 (2H, *m*)	32.2		1", 2", 3", 4", 6"
6"	1.49 (2H, *m*)	28.0		5", 7"
7"	4.02 (1H, br*t*, *J* = 6.6)	75.7	10"β	5", 9", 10"
8"	-	147.2		
9"	1.72 (3H, *s*)	17.9	10"α	7", 8", 10"
10"α	4.87 (1H, *s*)	111.2	9"	7", 8", 9"
10"β	4.94 (1H, *s*)		7"	7", 8", 9"
1'''	-	206.1		
2'''	3.18 (2H, *d*, *J* = 6.7)	53.4	3''', 4''', 5'''	1''', 3''', 4''', 5'''
3'''	2.31 (1H, *m*)	25.6	2''', 4''', 5'''	1''', 2''', 4''', 5'''
4'''	1.06 (6H, *d*, *J* = 6.7)	22.7	2''', 3'''	3''', 5'''
5'''		22.7	2''', 3'''	3''', 4'''

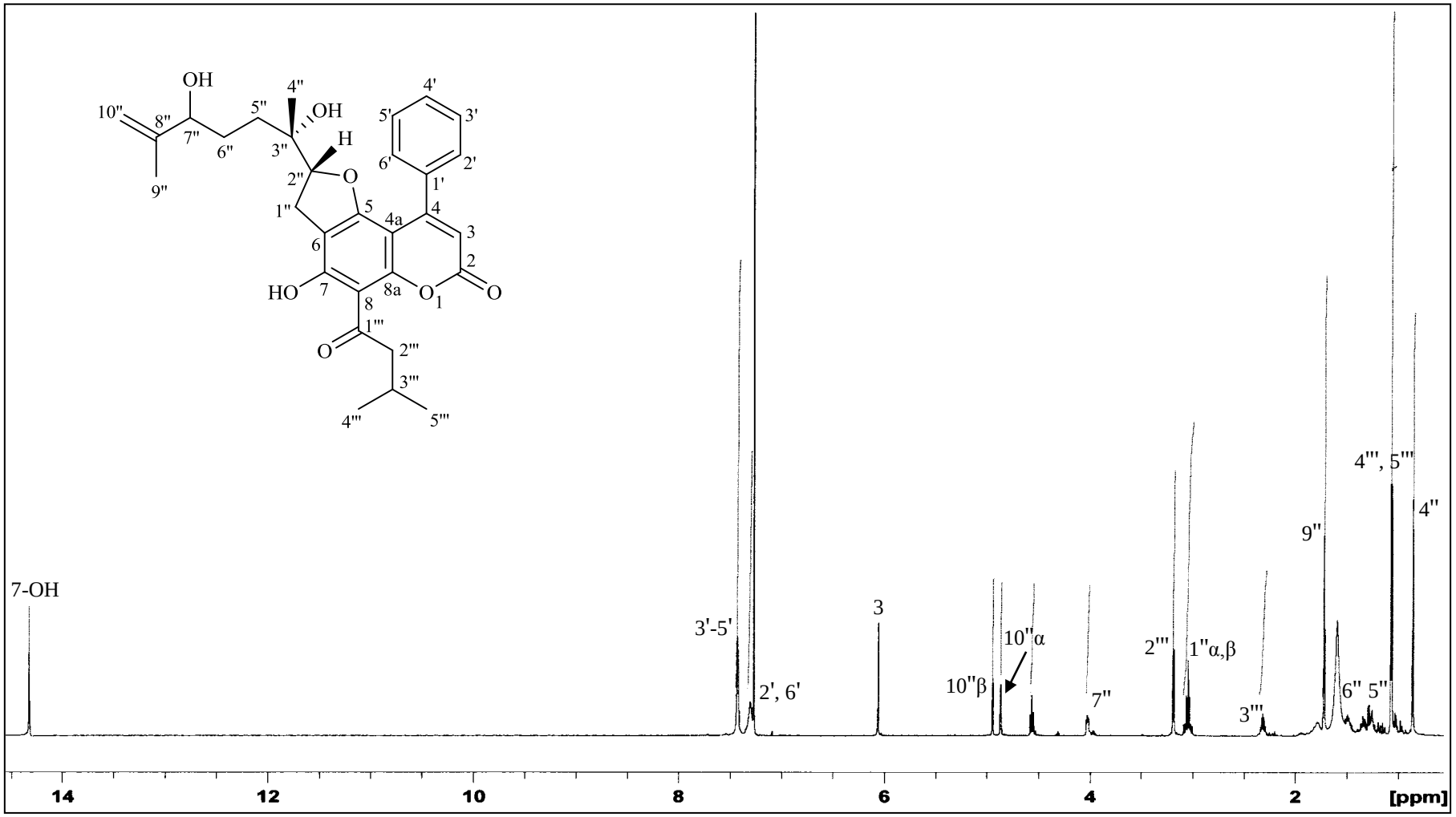


Figure 3.47: ^{1}H NMR Spectrum of Compound W **204**

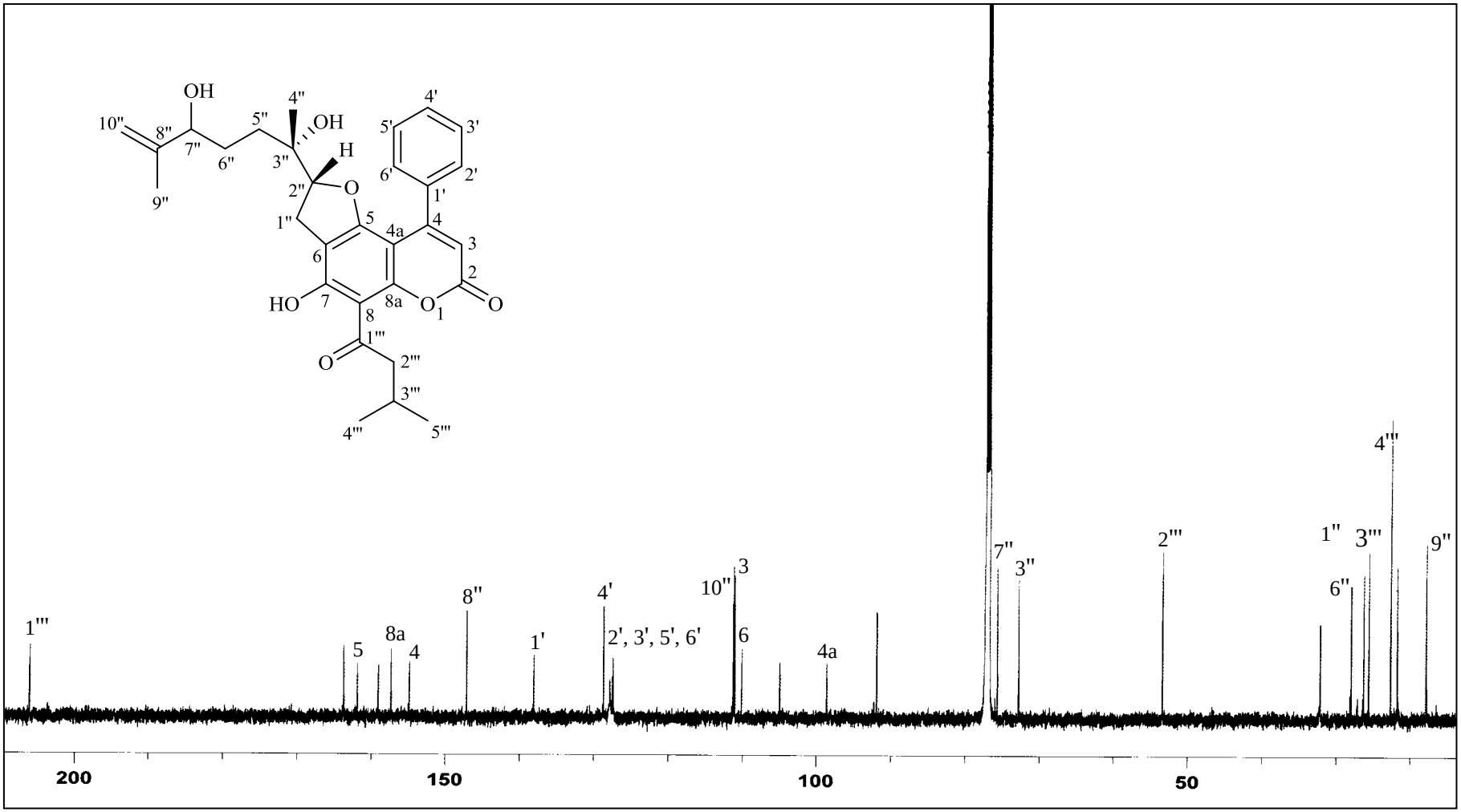
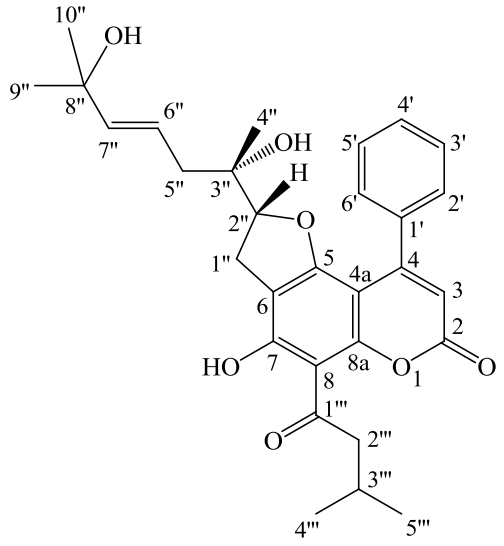


Figure 3.48: ^{13}C NMR Spectrum of Compound W **204**

3.1.24 Compound X: Mesuagenin N 205



205

Compound X was obtained as a yellowish oil and has a molecular formula of $C_{30}H_{34}O_7$ based on the HRESIMS measurement; $[M+Na]^+$ ion at *m/z* 529.2206 (calculated 529.2202). The IR spectrum showed strong absorptions at υ_{max} 3445 (free OH group), 1743 (α-pyrone), 1603 (chelated acyl group) and 1384 cm^{-1} (geminal dimethyl group).[47, 88]. The UV spectrum (EtOH) of compound X could be associated with an 8-acyl-5,7-dioxycoumarin type due to absorptions at λ_{max} 230, 285 and 339 nm[82].

A total of thirty carbons were observed in the ^{13}C NMR spectrum of compound X; five methyls, three methylenes, ten methines and twelve quaternary carbons. The ^1H NMR spectrum of compound X was similar to those of compound O, thus suggesting a close relationship between these compounds. The same substituents could be characterized in the two compounds, namely a typical H-3 signal of a 4-phenylcoumarin (δ 6.05, *s*, H-3), a monosubstituted phenyl (δ 7.43 and 7.31, 5H, *m*, H-2'-H6'), a chelated hydroxyl group (δ 14.31, *s*, 7-OH) and a 3-methylbutanoyl group [δ 3.17 (2H, *d*, *J* = 6.8 Hz, H-2'''), 2.30 (1H, *m*, H-3''') and 1.05 (6H, *d*, *J* = 6.8 Hz, H-4''' and H-5''')].

Close analysis of the ^{1}H and ^{13}C NMR spectra of compound X and compound O showed differences in the coupling patterns of the substituent group at position C-5/C-6. In fact, the existence of a double bond between C-6" and C-7" were observed with a different coupling pattern of H-6" and H-7" in the ^{1}H NMR spectrum of compound X as compared to compound O; δ 5.55 (1H, *m*, H-6") and 5.58 (1H, *d*, *J* = 15.6 Hz, H-7"). The manifestation of a strong singlet integrating for six protons at δ 1.29 made clear the presence of the H-9" and H-10" methyls in compound X. These observations showed that compound X has a slightly different hydroxylated prenyl group attached to C-5" as compared to compound O. Furthermore, the HMBC spectrum of compound X disclosed that this hydroxylated prenyl group was attached to C-5" (δ 40.1) by exhibiting the following correlations: H-6"/C-5", C-7" and C-8".

In the NOESY spectrum of compound X, cross correlation was observed between H-2" and H-4", which suggested that these two protons were located in the same plane.

The observed spectral data of compound X and a thorough search in the SciFinder database revealed that this compound was a new chemical entity, which was named as mesuagenin N **205**.

Table 3.25: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound X **205**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	159.1		
3	6.05 (1H, *s*)	111.2		2, 4a, 1'
4	-	155.0		
4a	-	98.8		
5	-	162.0		
6	-	110.1		
7-OH	14.31 (1H, *s*)	163.8		6, 7, 8, 1'''
8	-	105.1		
8a	-	157.4		
1'	-	138.2		
2'	7.31 (1H, *m*, Ar)	127.5		4, 4'
3'	7.43 (3H, *m*, Ar)	128.0		1'
4'		128.9		2', 6'
5'		128.0		1'
6'	7.31 (1H, *m*, Ar)	127.5		4, 4'
1''	3.03 (2H, *d*, *J* = 9.3)	26.3	2''	5, 6, 7, 2'', 3''
2''	4.52 (1H, *t*, *J* = 9.3)	91.5	1''	5, 6, 4'', 5''
3''	-	73.1		
4''	0.85 (3H, *s*)	22.2		2'', 3'', 5''
5''	1.97 (2H, *m*)	40.1	6''	2'', 3'', 4'', 6'', 7''
6''	5.55 (1H, *m*)	120.5	5''	5'', 7'', 8''
7''	5.58 (1H, *d*, *J* = 15.6)	142.8		6'', 9'', 10''
8''	-	70.8		
9''	1.29 (3H, *s*)	29.9		6'', 7'', 8''
10''	1.29 (3H, *s*)	29.9		6'', 7'', 8''
1'''	-	206.1		
2'''	3.17 (2H, *d*, *J* = 6.8)	53.5	3'''	1''', 3''', 4''', 5'''
3'''	2.30 (1H, *m*)	25.7	2''', 4''', 5'''	1''', 2''', 4''', 5'''
4'''	1.05 (6H, *d*, *J* = 6.8)	22.8	3''', 5'''	2''', 3''', 5'''
5'''		22.8	3''', 4'''	2''', 3''', 4'''

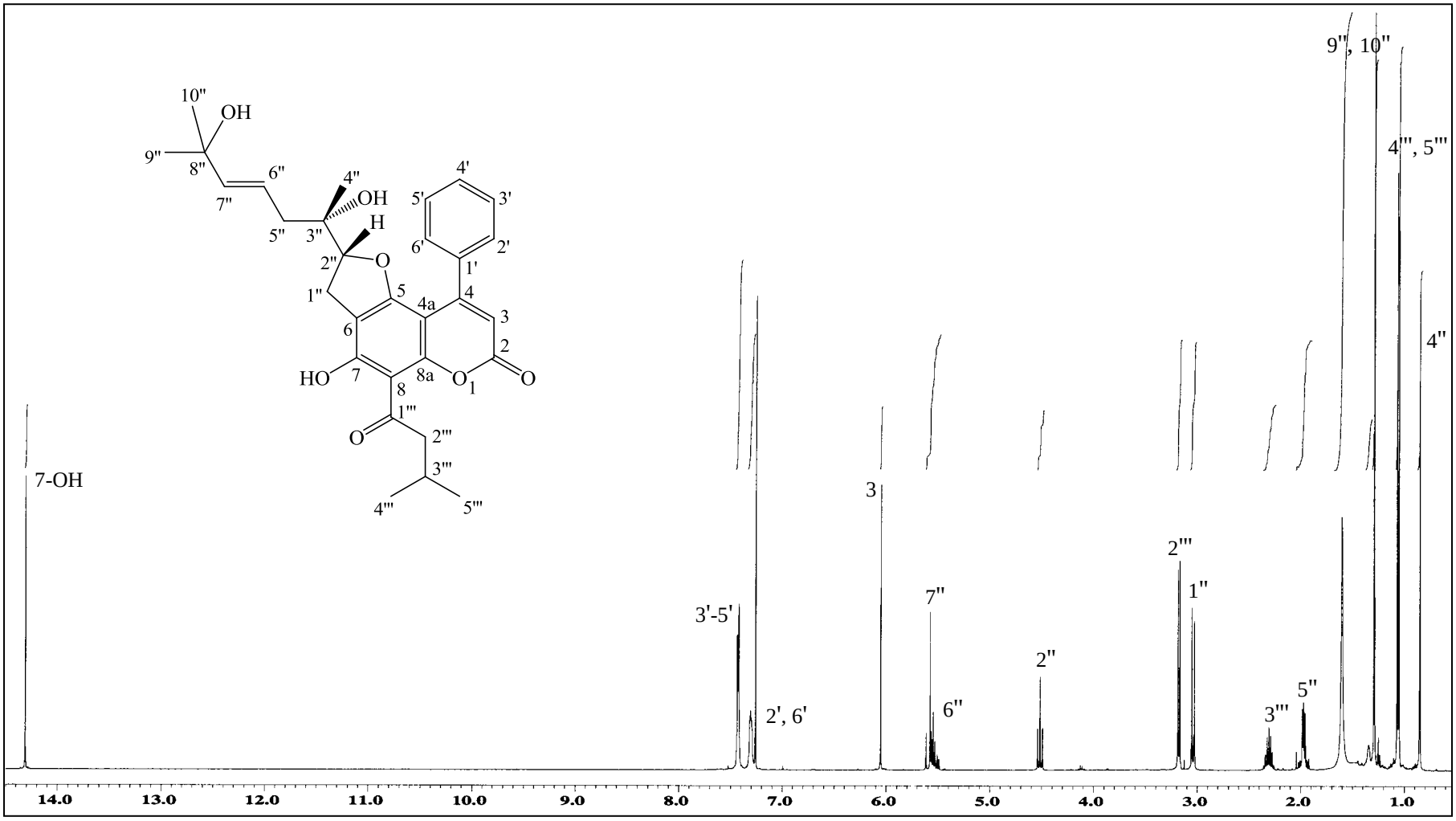


Figure 3.49: ^{1}H NMR Spectrum of Compound X **205**

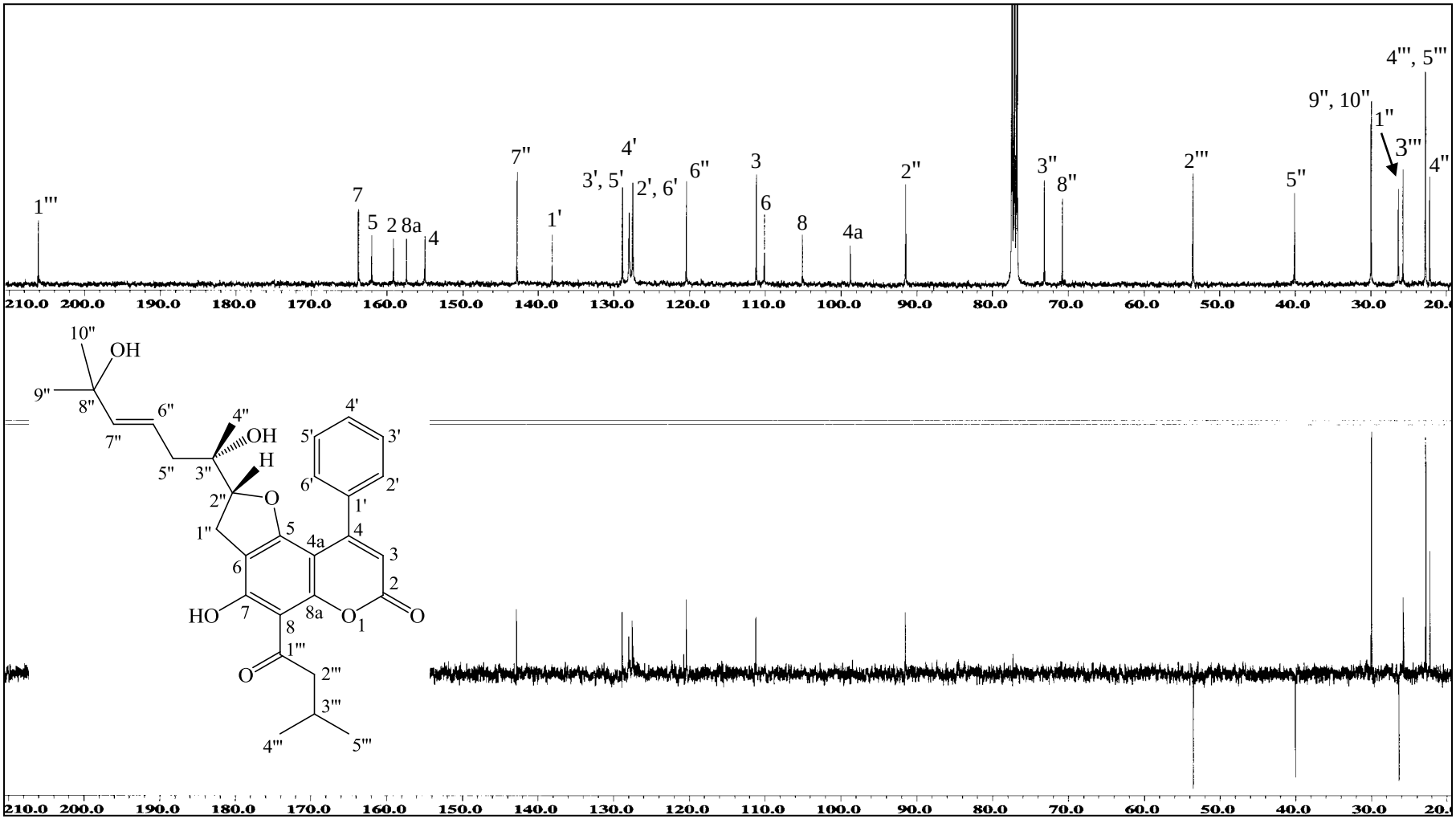


Figure 3.50: ^{13}C NMR and DEPT135 Spectra of Compound X **205**

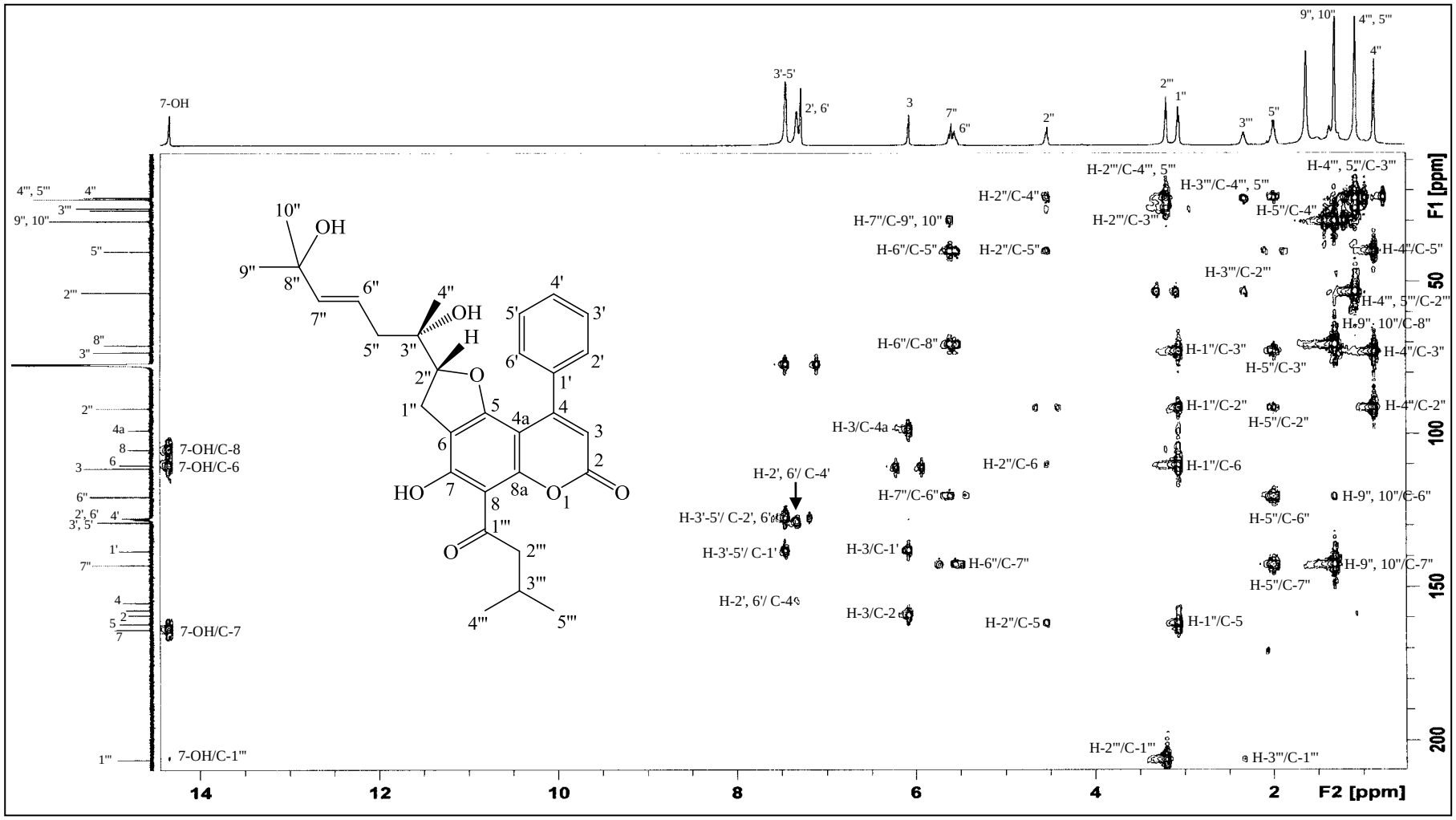


Figure 3.51: HMBC Spectrum of Compound X **205**

CHAPTER 4

SEMI-SYNTHESIS OF 4-PHENYLCOUMARIN ANALOGUES

The present chapter deals with the structural elucidation of seventeen semi-synthesized 4-phenylcoumarin analogues from *Mesua elegans* (King) Kosterm. Three types of semi-synthetic work were carried out successfully, namely acetylation, methylation and bromobenzoylation. These work were done, to evaluate the effects of hydroxyl group in the AChE inhibitory activity and SAR studies, which shall be discussed in chapter 5 later. The following sections describe the fractions or the parent compounds involved in the semi-synthesis work, and the respected compounds isolated from these semi-synthetic studies.

4.1 Semi-synthesis of 4-Phenylcoumarin Analogues

Fractions 1 and 1a from the hexane extract of *M. elegans* were subjected to three types of chemical modifications; acetylation, methylation and benzoylation. The findings are discussed briefly in the following sections.

4.1.1 Acetylation

Fraction F1 (hexane:ethyl acetate 95:5) from the hexane extract of the bark of *Mesua elegans* was subjected to the acetylation process. This fraction essentially contained five chemical constituents; compound F (mammea A/BB) **55**, compound G (mammea A/BA) **28**, compound H (mesuagenin C) **191**, compound I [5,7-dihydroxy-8-(2-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one (DMDP-1)] **47** and compound J [5,7-dihydroxy-8-(3-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one (DMDP-2)] **48**. The semi-

synthesized compounds were isolated from this fraction and the following Table 4.1 shows the names of the isolates.

Table 4.1: Acetylation Isolates and Their Names.

Acetylation Isolates	Names of Isolates
Compound A1	7-hydroxy-8-(2-methylbutanoyl)-6-(3-methylbut-2-enyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl acetate **206**
Compound A2	7-hydroxy-8-(3-methylbutanoyl)-6-(3-methylbut-2-enyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl acetate **207**
Compound A3	6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-7-hydroxy-8-(2-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl acetate **208**
Compound A4	6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-7-hydroxy-8-(3-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl acetate **209**
Compound A5	8-(2-methylbutanoyl)-6-(3-methylbut-2-enyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl diacetate **210**
Compound A6	8-(3-methylbutanoyl)-6-(3-methylbut-2-enyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl diacetate **211**
Compound A7	6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-8-(isobutyryl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl diacetate **212**
Compound A8	6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-8-(2-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl diacetate **213**
Compound A9	6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-8-(3-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl diacetate **214**

4.1.2 Methylation

Methylation was carried out using fraction F1 (hexane:ethyl acetate 95:5) from the hexane extract of the bark of *Mesua elegans*. The parent compounds F - J in this fraction were methylated to afford compounds M1 - M4. Table 4.2 depicts the isolates of the methylation process.

Table 4.2: Methylation Isolates and Their Names.

Methylation Isolates	Names of Isolates
Compound M1	5,7-dimethoxy-8-(2-methylbutanoyl)-6-(3-methylbut-2-enyl)-4-phenyl-2*H*-chromen-2-one **215**
Compound M2	5,7-dimethoxy-6-[(*E*)-(3,7-dimethylocta-2,6-dienyl]-8-isobutyryl-4-phenyl-2*H*-chromen-2-one **216**
Compound M3	5,7-dimethoxy-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-8-(2-methylbutanoyl)-4-phenyl-2*H*-chromen-2-one **217**
Compound M4	5,7-dimethoxy-6-[(*E*)-(3,7-dimethylocta-2,6-dienyl]-8-(3-methylbutanoyl)-4-phenyl-2*H*-chromen-2-one **218**

4.1.3 Bromobenzoylation

Fraction F1-2 from the hexane extract of the bark of *Mesua elegans* was been subjected to the bromobenzoylation process. This fraction contained two major chemical constituents; compound I [5,7-dihydroxy-8-(2-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one (DMDP-1)] **47** and compound J [5,7-dihydroxy-8-(3-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one (DMDP-2)] **48**. Table 4.3 below designates the isolates from the bromobenzoylation reaction.

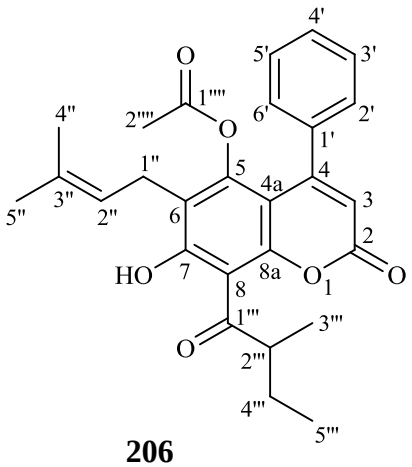
Table 4.3: Bromobenzoylation Isolates and Their Names.

Bromobenzoylation Isolates	Names of Isolates
Compound B1	(*E*)-6-(3,7-dimethylocta-2,6-dienyl)-7-hydroxy-8-(3-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl 2-bromobenzoate **219**
Compound B2	(*E*)-6-(3,7-dimethylocta-2,6-dienyl)-7-hydroxy-8-(2-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl 2-bromobenzoate **220**
Compound B3	(*E*)-6-(3,7-dimethylocta-2,6-dienyl)-8-(3-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl bis(2-bromobenzoate) **221**
Compound B4	(*E*)-6-(3,7-dimethylocta-2,6-dienyl)-8-(2-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl bis(2-bromobenzoate) **222**

4.2 Structural Elucidation of Compounds from the Semi-synthesis of 4-Phenylcoumarin Analogues

All the semi-synthesized compounds were subjected to isolation using chromatographic techniques, including preparative thin layer chromatography (PTLC) and high performance liquid chromatography (HPLC). The isolates were then elucidated using spectroscopic techniques, namely 1D and 2D NMR, IR and UV. The following sub-chapters explain the structural elucidation of these compounds.

4.2.1 Compound A1: 7-Hydroxy-8-(2-methylbutanoyl)-6-(3-methylbut-2-enyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl acetate 206



206

Compound A1 was acetylated in fraction F1 (hexane:ethyl acetate 95:5) of the hexane extract of *M. elegans*. Compound A1 was then isolated by PTLC and HPLC as a yellow oil. The HRESIMS spectrum exhibited pseudomolecular $[M+Na]^+$ ion at *m/z* 471.1496 (calculated 471.1784), which suggested a molecular formula of $C_{27}H_{28}O_6$. The UV spectrum (EtOH) of compound A1 showed peaks at 221, 295 and 330 nm, which showed a resemblance with the UV absorbance of the parent compound F (226, 297, 331 nm), supporting the same skeletal structure. The IR spectrum showed absorption at υ_{max} 3480 cm^{-1}, indicating the presence of hydroxyl group, which was apparent in the parent compound F. The IR spectrum of compound A1 also exhibited absorption at υ_{max} 1735 cm^{-1}, which showed the presence of α-pyrone and ester groups.

The ^{13}C NMR spectrum showed twenty seven carbon signals (five methyls, two methylenes, eight methines and twelve quaternary carbons), which was an addition of two carbons compared to the parent compound F, which has twenty five carbons. In the ^1H NMR spectrum of compound A1, the proton signal for the 5-OH which was apparent in the parent compound F's ^1H NMR spectrum at δ 5.94 was missing, indicating that acetylation had taken place at position 5-OH of the parent compound F. In addition, a methyl signal was apparent in the ^1H NMR spectrum of compound A1, at δ 1.26 (3H, *s*,

H-2""), which was absent in ^{1}H NMR spectrum of the parent compound F, suggesting the presence of the an acetyl group in compound A1.

The observed data of compound A1 suggested that this was an acetylated derivative of the parent compound F at position C-5, with the IUPAC name 7-hydroxy-8-(2-methylbutanoyl)-6-(3-methylbut-2-enyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl acetate **206**.

Table 4.4: ^{1}H NMR and ^{13}C NMR (in $CDCl_3$, 400 MHz) of Compound A1 **206**

Position	δ_H, *J* (Hz)	δ_C
2	-	158.4
3	6.05 (1H, *s*)	114.3
4	-	154.4
4a	-	105.3
5	-	150.2
6	-	121.3
7-OH	14.36 (1H, *s*)	166.4
8	-	107.8
8a	-	154.8
1'	-	138.3
2'	7.31 (1H, *m*, Ar)	127.8
3'	7.44 (3H, *m*, Ar)	128.3
4'		128.7
5'		128.3
6'	7.31 (1H, *m*, Ar)	127.8
1"	3.12 (1H, *brd*, *J* = 42.5)	23.0
2"	4.98 (1H, *brt*, *J* = 6.8)	120.4
3"	-	133.0
4"	1.67 (3H, *s*)	17.9
5"	1.63 (3H, *s*)	25.6
1'"	-	211.4
2'"	3.97 (1H, *sext*, *J* = 6.8)	47.5
3'"	1.29 (3H, *d*, *J* = 6.8)	16.6
4'"α	1.49 (1H, *m*)	27.1
4'"β	1.96 (1H, *m*)	
5'"	1.02 (3H, *t*, *J* = 7.3)	11.8
1""	-	168.3
2""	1.26 (3H, *s*)	19.1

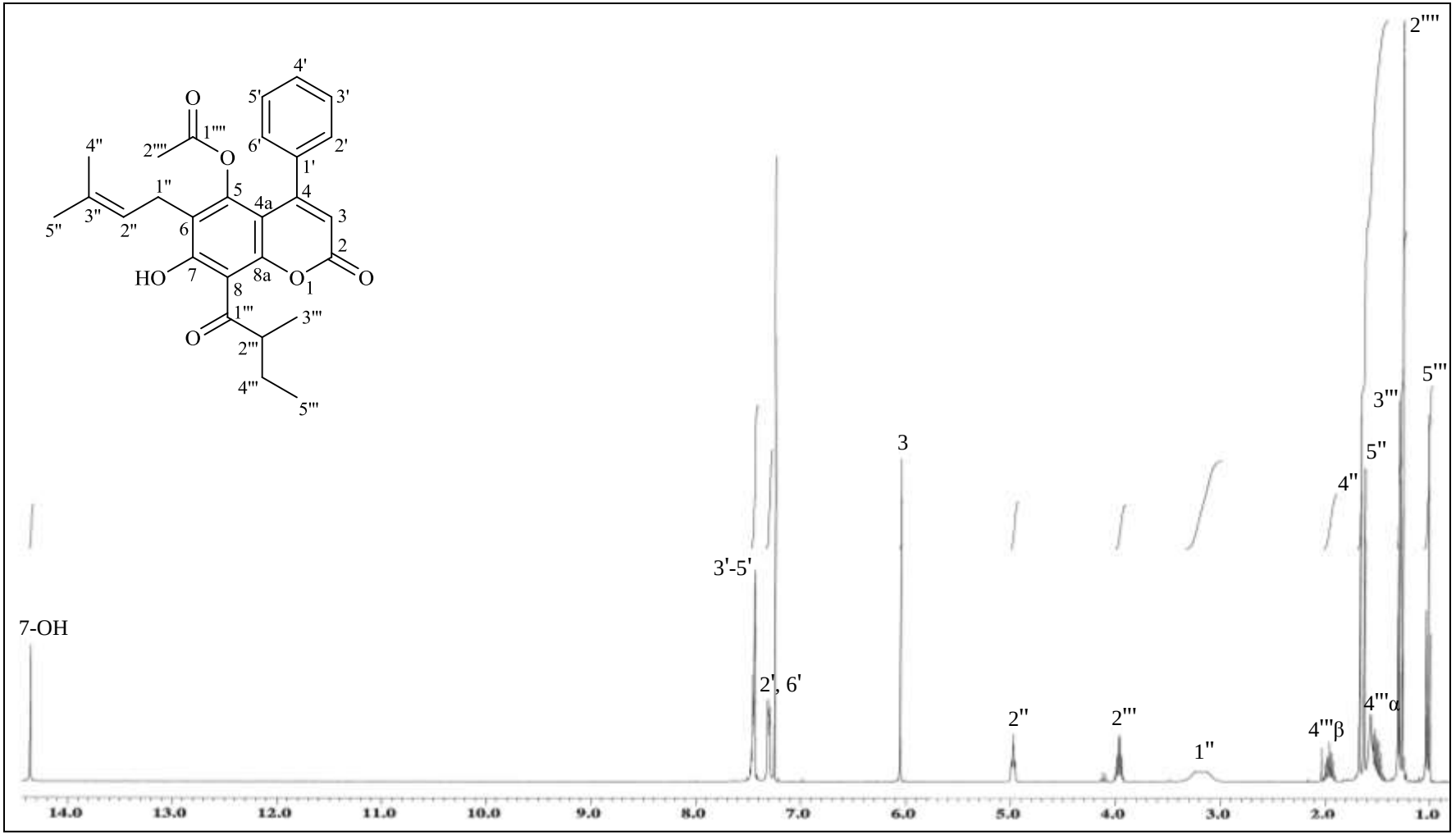


Figure 4.1: ^{1}H NMR Spectrum of Compound A1 **206**

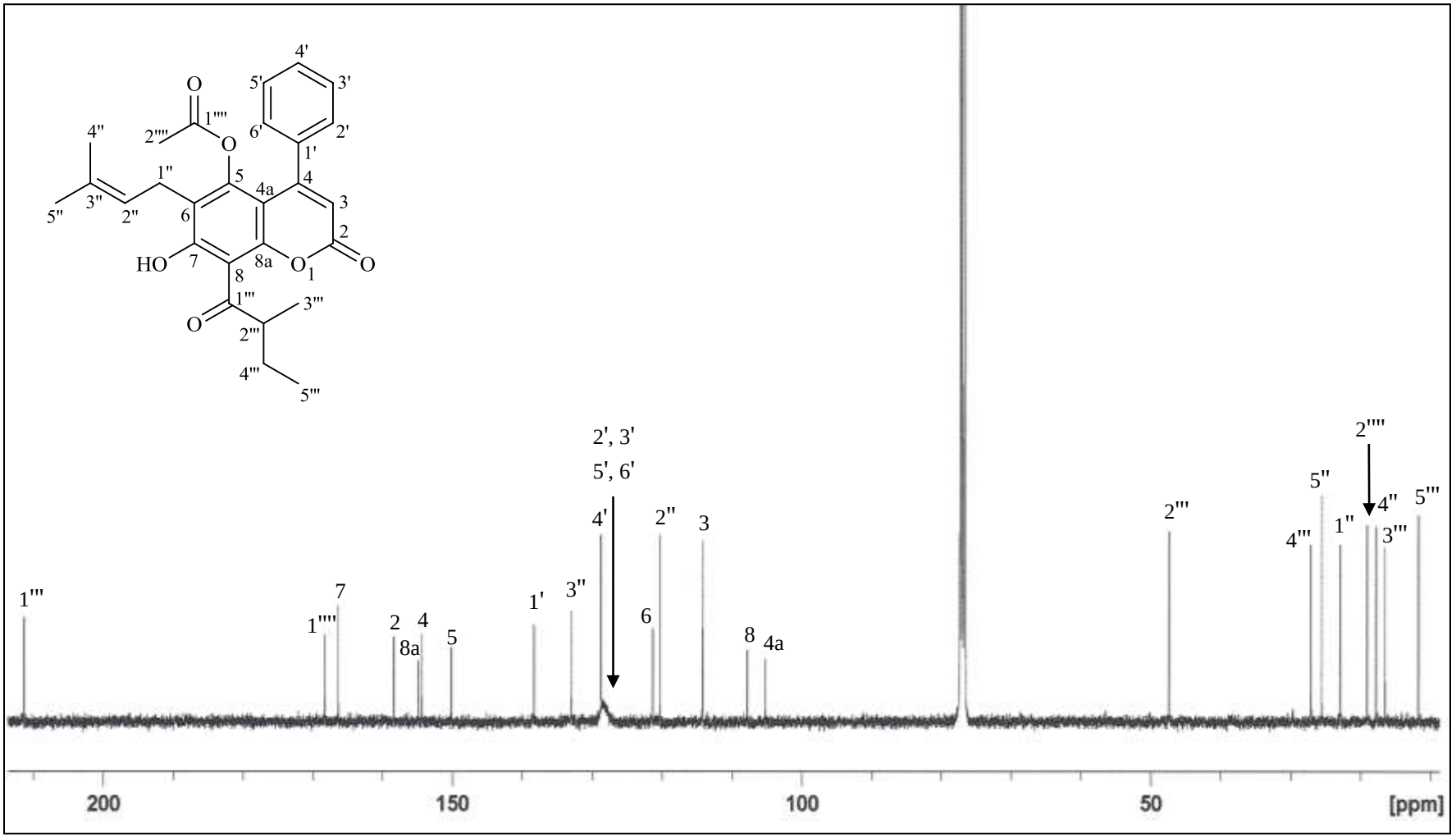
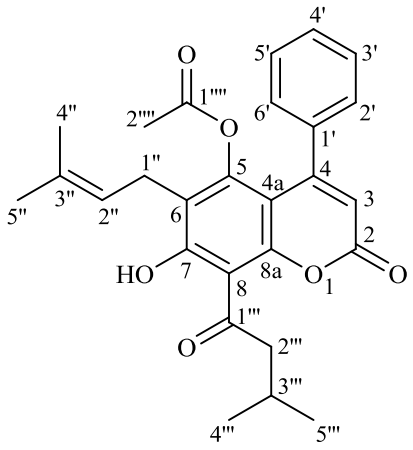


Figure 4.2: ^{13}C NMR Spectrum of Compound A1 **206**

4.2.2 Compound A2: 7-Hydroxy-8-(3-methylbutanoyl)-6-(3-methylbut-2-enyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl acetate 207



207

Compound A2 was isolated as a yellow oil after being acetylated in fraction F1 (hexane:ethyl acetate 95:5) of the hexane extract of *M. elegans*, in which compound G was the parent compound within this fraction. The HRESIMS spectrum displayed pseudomolecular $[M+Na]^+$ ion at *m/z* 471.1549 (calculated 471.1784), suggesting a molecular formula of $C_{27}H_{28}O_6$. The IR spectrum showed absorptions at υ_{max} 1735 cm^{-1} (α, β-unsaturated lactone and ester groups), which were similar to those in the parent compound G. The UV spectrum (EtOH) of compound A2 also supported the same skeletal structure as compound G, by exhibiting peaks at 225, 297 and 333 nm.

A total of twenty-seven carbon resonances were observed in the ^{13}C NMR spectrum of compound A2 (five methyls, two methylenes, eight methines and twelve quaternary carbons), as compared to the parent compound G which has twenty five carbons. In the ^1H NMR spectrum of compound A2, an additional methyl signal at δ 1.26 (3H, *s*, H-2'''') was noticeable, which was absent in the parent compound G. On the other hand, the 5-OH signal at δ 5.95 in the ^1H NMR spectrum of compound G was missing in the ^1H NMR spectrum of compound A2. This indicated that the acetylation had taken place at

position C-5 of the coumarin skeleton of compound A2. Furthermore, hydroxyl group of C-7 has a strong hydrogen bond with the neighbouring ketone group (C-1'"), therefore making this hydroxyl group more difficult to be acetylated.

Comparison of the spectral data between compound A2 and the parent compound G indicated that this was an acetylated derivative of the parent compound G at position C-5, with the IUPAC name 7-hydroxy-8-(3-methylbutanoyl)-6-(3-methylbut-2-enyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl acetate **207**.

Table 4.5: ^{1}H NMR and ^{13}C NMR (in $CDCl_3$, 400 MHz) of Compound A2 **207**

Position	δ_H, *J* (Hz)	δ_C
2	-	158.4
3	6.05 (1H, *s*)	114.4
4	-	154.4
4a	-	105.3
5	-	150.3
6	-	121.4
7-OH	14.42 (1H, *s*)	166.3
8	-	108.2
8a	-	155.1
1'	-	138.3
2'	7.32 (1H, *m*, Ar)	127.9
3'	7.45 (3H, *m*, Ar)	128.4
4'		128.8
5'		128.4
6'	7.32 (1H, *m*, Ar)	127.9
1"	3.12 (1H, br*d*, *J* coupling obscured by peak H-2"')	23.0
2"	4.97 (1H, br*t*, *J* = 6.8)	120.4
3"	-	133.1
4"	1.67 (3H, *s*)	17.9
5"	1.63 (3H, *s*)	25.7
1"'	-	207.0
2"'	3.23 (2H, *d*, *J* = 6.8)	54.0
3"'	2.35 (1H, *m*)	25.4
4"'	1.05 (6H, *d*, *J* = 6.8)	22.7
5"'		22.7
1""	-	168.5
2""	1.26 (3H, *s*)	19.2

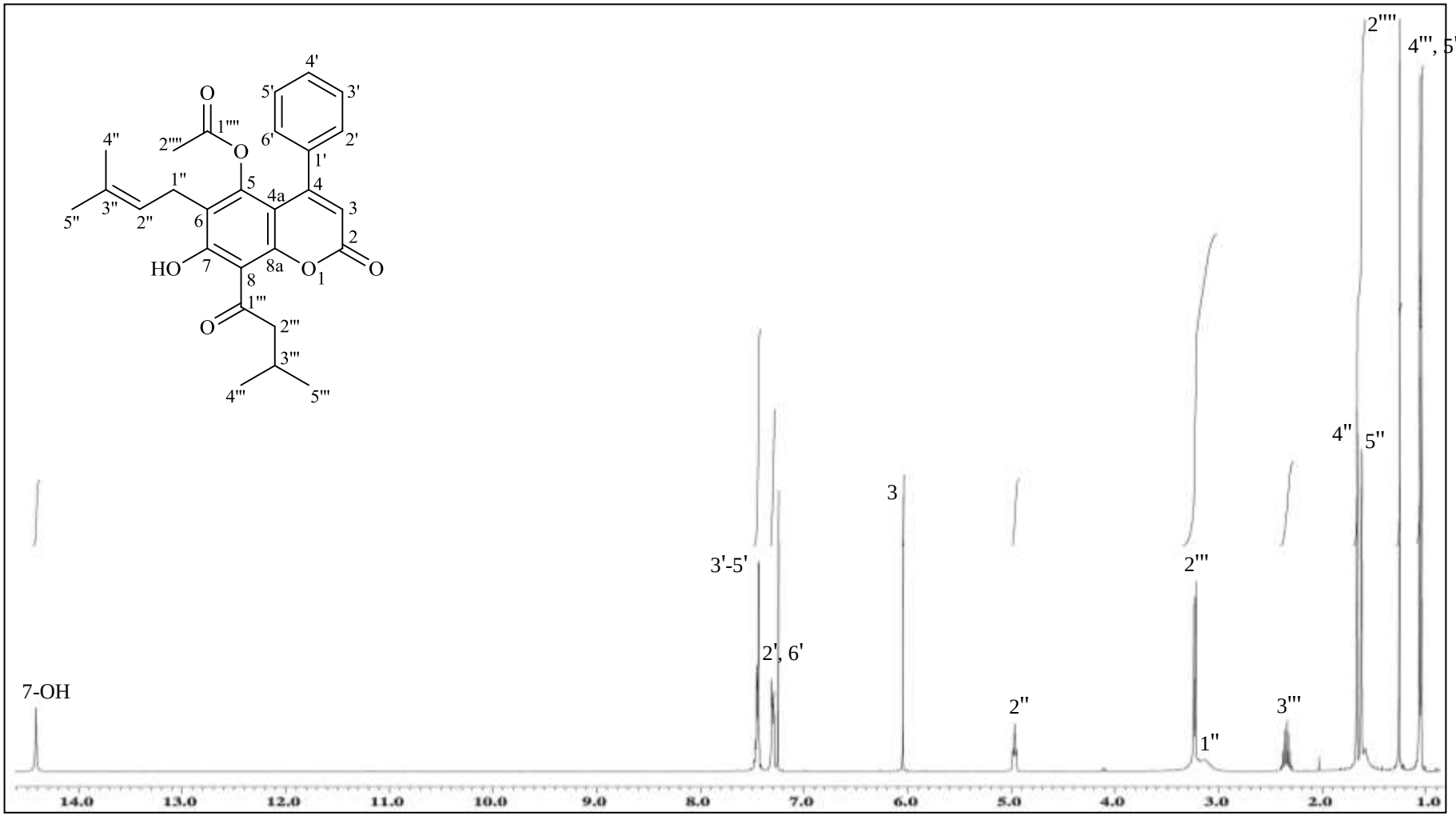
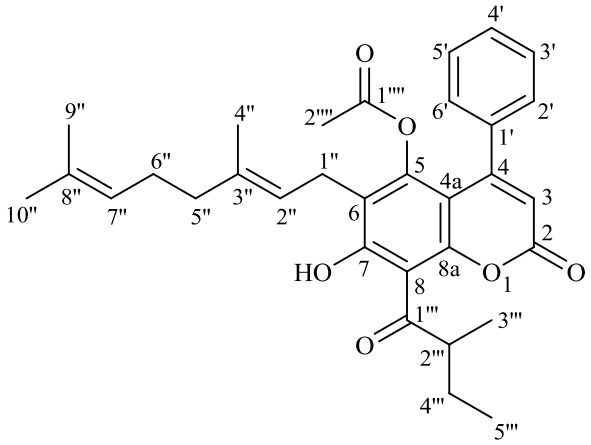


Figure 4.3: ^{1}H NMR Spectrum of Compound A2 **207**

4.2.3 Compound A3: 6-[(*E*)-3,7-Dimethylocta-2,6-dienyl]-7-hydroxy-8-(2-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl acetate 208



208

Fraction F1 (hexane:ethyl acetate 95:5) of the hexane extract of *M. elegans* was subjected to acetylation, and compound A3 was isolated from the product of this semi-synthetic process through PTLC and HPLC as a yellow oil. The HRESIMS spectrum exhibited a pseudomolecular $[M+Na]^+$ ion at *m/z* 539.2714 (calculated 539.2410), suggesting a molecular formula of $C_{32}H_{36}O_6$. The IR spectrum showed absorptions at υ_{max} 3452 cm^{-1} (hydroxyl group), 1739 cm^{-1} (α-pyrone and ester groups) and 1600 cm^{-1} (chelated acyl group), comparable to those in the parent compound I. The UV absorbance of compound A3 was similar to the parent compound I, with intense absorption bands at 225, 295 and 334 nm.

The ^{13}C NMR spectrum of compound A3 showed an addition of two carbons compared to the parent compound I, with a total of thirty-two carbon signals (six methyls, four methylenes, nine methines and thirteen quaternary carbons). In the ^1H NMR spectrum of compound A3, the proton signal for the 5-OH which was apparent in the ^1H NMR spectrum of compound I at δ 5.94 was missing, indicating that the acetylation took place at position C-5 of the parent compound I. In addition, a methyl signal was apparent in

the ^{1}H NMR spectrum of compound A3, at δ 1.26 (3H, *s*, H-2''''), which was absent in the ^{1}H NMR spectrum of parent compound I, thus suggesting the presence of the acetyl group in compound A3.

The observed data of compound A3 suggested that this was an acetylated derivative of the parent compound I at position C-5, with the IUPAC name 6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-7-hydroxy-8-(2-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl acetate **208**.

Table 4.6: ^{1}H NMR and ^{13}C NMR (in $CDCl_3$, 400 MHz) of Compound A3 **208**

Position	δ_H, *J* (Hz)	δ_C
2	-	158.5
3	6.05 (1H, *s*)	114.3
4	-	154.4
4a	-	105.4
5	-	150.3
6	-	121.5
7-OH	14.30 (1H, *s*)	166.5
8	-	107.9
8a	-	154.9
1'	-	138.4
2'	7.31 (1H, *m*, Ar)	127.5
3'	7.44 (3H, *m*, Ar)	128.5
4'		128.8
5'		128.5
6'	7.31 (1H, *m*, Ar)	127.5
1"	3.17 (2H, brs)	22.9
2"	4.97 (1H, br*t*, *J* = 6.7)	120.3
3"	-	136.6
4"	1.66 (3H, *s*)	16.3
5"	1.93 (2H, *m*)	39.5
6"	1.98 (2H, *m*)	26.6
7"	5.02 (1H, br*t*, *J* = 6.7)	124.1
8"	-	131.5
9"	1.55 (3H, *s*)	17.7
10"	1.62 (3H, *s*)	25.7
1"'	-	211.5
2"'	3.96 (1H, *sext*, *J* = 6.3)	47.5
3"'	1.29 (3H, *d*, *J* = 6.3)	16.6
4"'α	1.49 (1H, *m*)	27.2
4"'β	1.89 (1H, *m*)	
5"'	1.02 (3H, *t*, *J* = 7.3)	11.9
1""	-	168.4
2""	1.26 (3H, *s*)	19.2

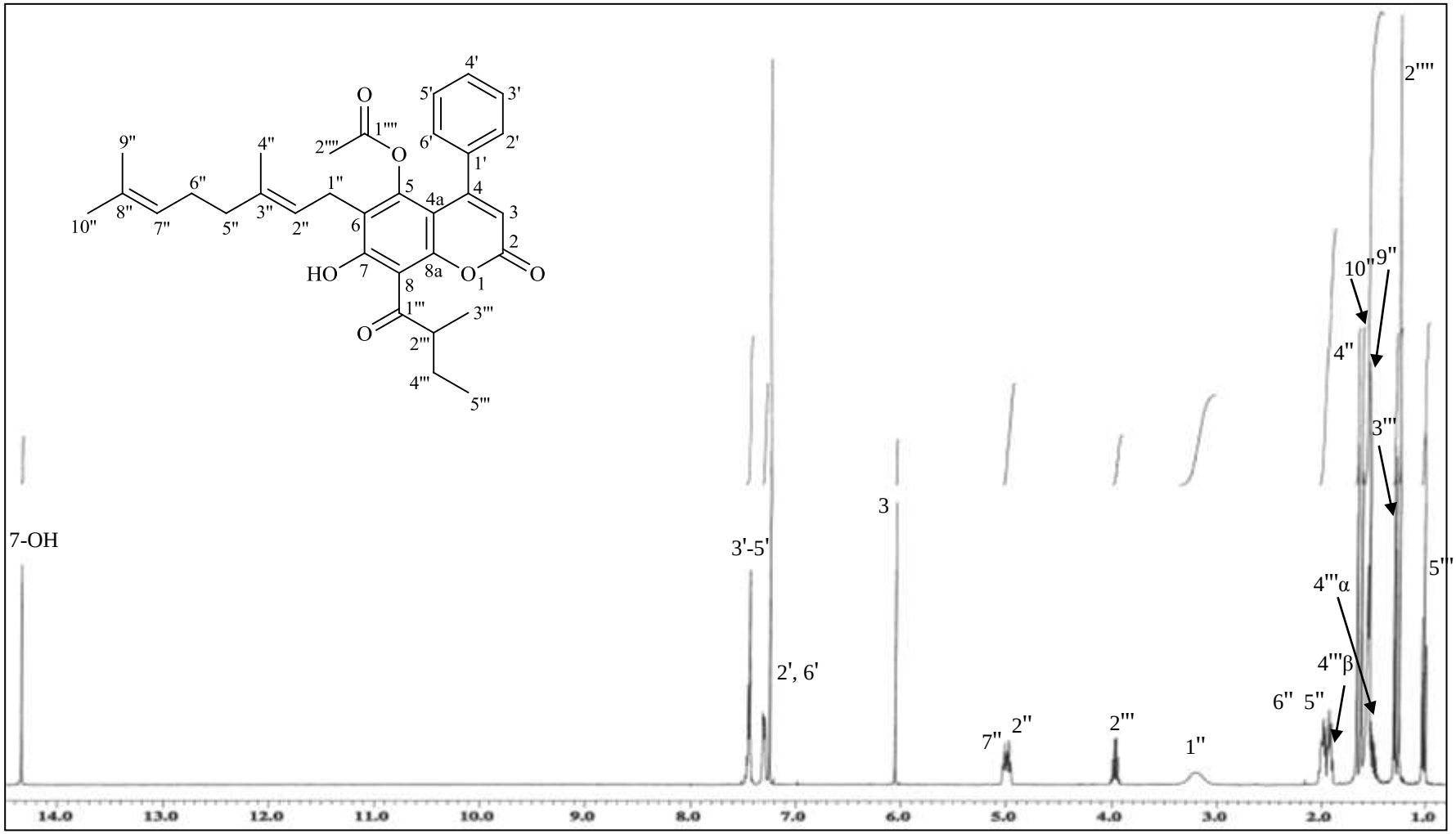


Figure 4.4: ^{1}H NMR Spectrum of Compound A3 **208**

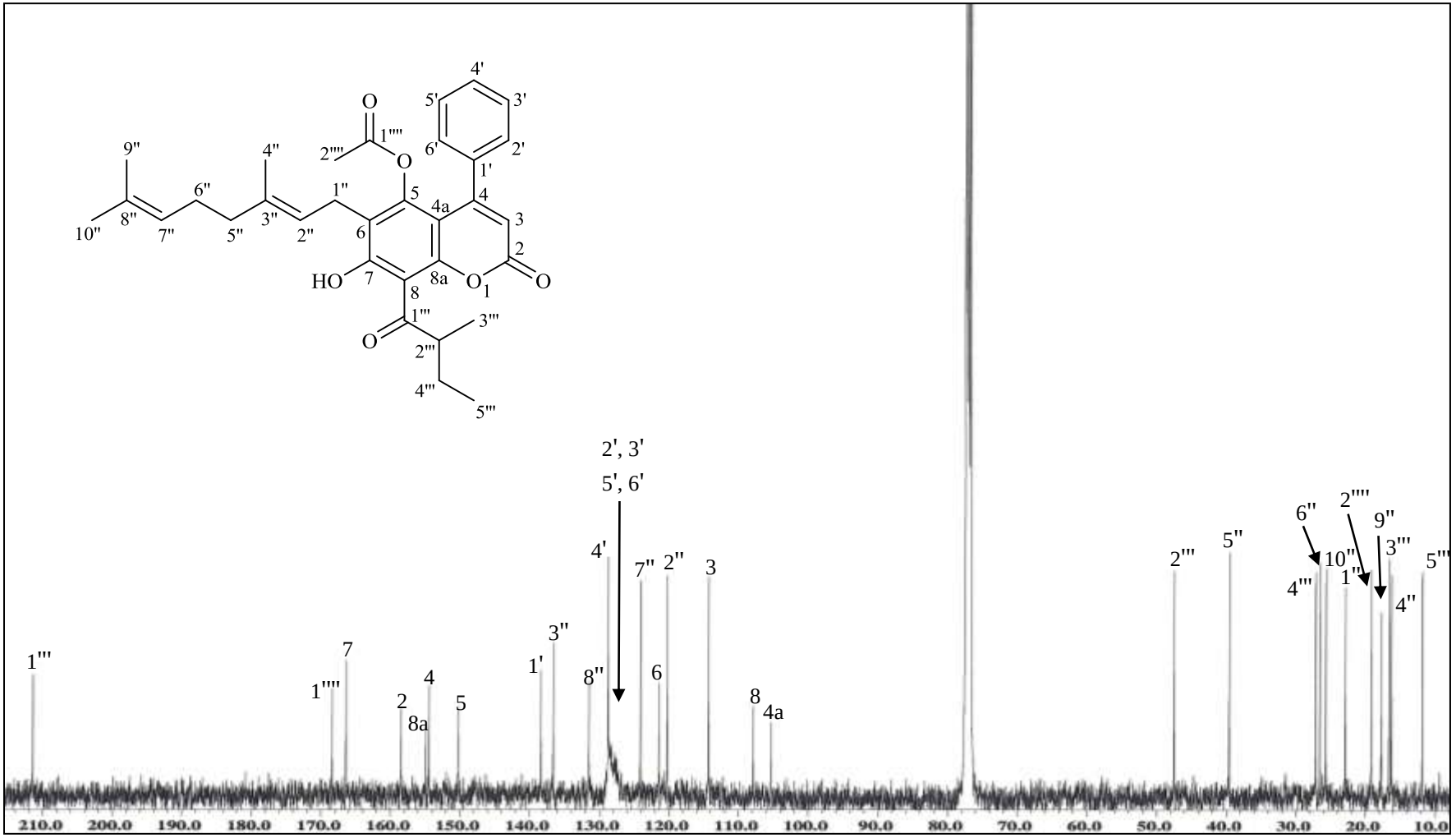
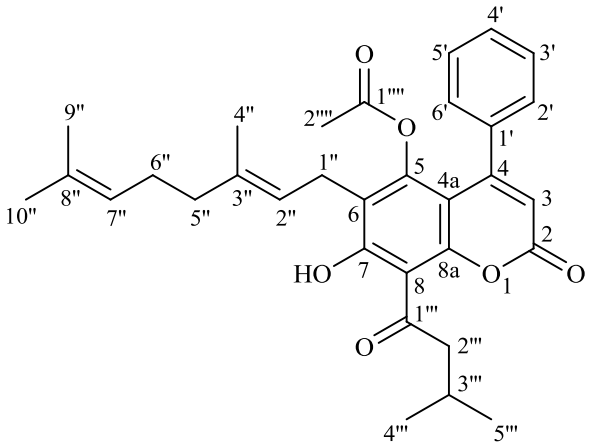


Figure 4.5: ^{13}C NMR Spectrum of Compound A3 **208**

4.2.4 Compound A4: 6-[(*E*)-3,7-Dimethylocta-2,6-dienyl]-7-hydroxy-8-(3-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl acetate 209



209

Compound A4 was an acetylated derivative of the parent compound J and was isolated as a white amorphous powder. The HRESIMS spectrum revealed a pseudomolecular $[M+Na]^+$ ion at *m/z* 539.2690 (calculated 539.2410), suggesting a molecular formula of $C_{32}H_{36}O_6$. The UV spectrum showed maximum absorption bands at 221, 297 and 333 nm, similar to the parent compound J (227, 299 and 332 nm), indicating the same skeletal structure. The IR spectrum showed absorptions at υ_{max} 3470 cm^{-1}, indicating the presence of a hydroxyl group. The IR spectrum of compound A4 also exhibited peaks at υ_{max} 1749 cm^{-1}, which showed the presence of α,β-unsaturated lactone and ester groups.

The ^{13}C NMR spectrum of compound A4 showed thirty-two carbon signals (six methyls, four methylenes, nine methines and thirteen quaternary carbons), which represents the addition of two new carbons as compared to the parent compound J. In the ^1H NMR spectrum of compound A4, an additional methyl signal was apparent at δ 1.27 (3H, *s*, H-2''''). On the other hand, signal of 5-OH was missing in compound A4 as compared to the ^1H NMR spectrum of parent compound J. This suggested that acetylation had occurred at C-5 in compound A4. Apart from these differences in the ^1H

NMR spectrum of compound A4, the proton signals for the other substituents (4-phenyl, geranyl and 3-methylbutanoyl) remained similar.

The observed data of compound A4 suggested that this was an acetylated derivative of the parent compound J at position C-5, with the IUPAC name 6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-7-hydroxy-8-(3-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl acetate **209**.

Table 4.7: ^{1}H NMR and ^{13}C NMR (in $CDCl_3$, 400 MHz) of Compound A4 **209**

Position	δ_H, *J* (Hz)	δ_C
2	-	158.3
3	6.06 (1H, *s*)	114.3
4	-	154.3
4a	-	105.3
5	-	150.3
6	-	121.3
7-OH	14.43 (1H, *s*)	166.3
8	-	108.2
8a	-	155.0
1'	-	138.3
2'	7.31 (1H, *m*, Ar)	127.8
3'	7.44 (3H, *m*, Ar)	128.3
4'		128.7
5'		128.3
6'	7.31 (1H, *m*, Ar)	127.8
1"	3.17 (2H, brs)	22.8
2"	4.98 (1H, br*t*, *J* = 6.7)	120.2
3"	-	136.6
4"	1.68 (3H, *s*)	16.2
5"	1.90 (2H, *m*)	39.6
6"	2.09 (2H, *m*)	26.5
7"	5.02 (1H, br*t*, *J* = 6.7)	124.1
8"	-	131.4
9"	1.56 (3H, *s*)	17.6
10"	1.64 (3H, *s*)	25.7
1'''	-	206.9
2'''	3.25 (2H, *d*, *J* = 6.6)	54.0
3'''	2.37 (1H, *sext*, *J* = 6.6)	25.4
4'''	1.06 (6H, *d*, *J* = 6.6)	22.7
5'''		22.7
1''''	-	168.3
2''''	1.27 (3H, *s*)	19.1

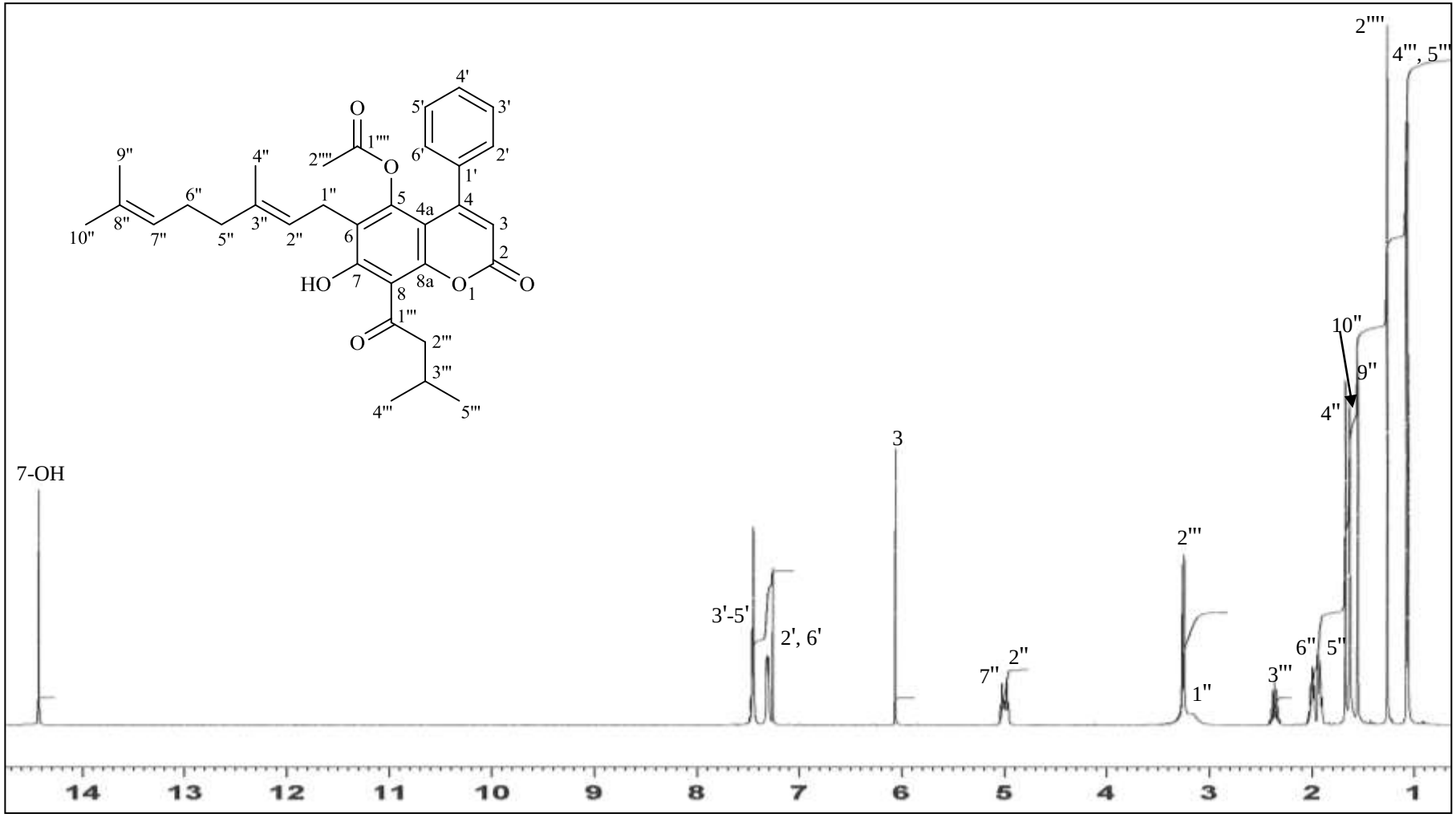


Figure 4.6: ^{1}H NMR Spectrum of Compound A4 **209**

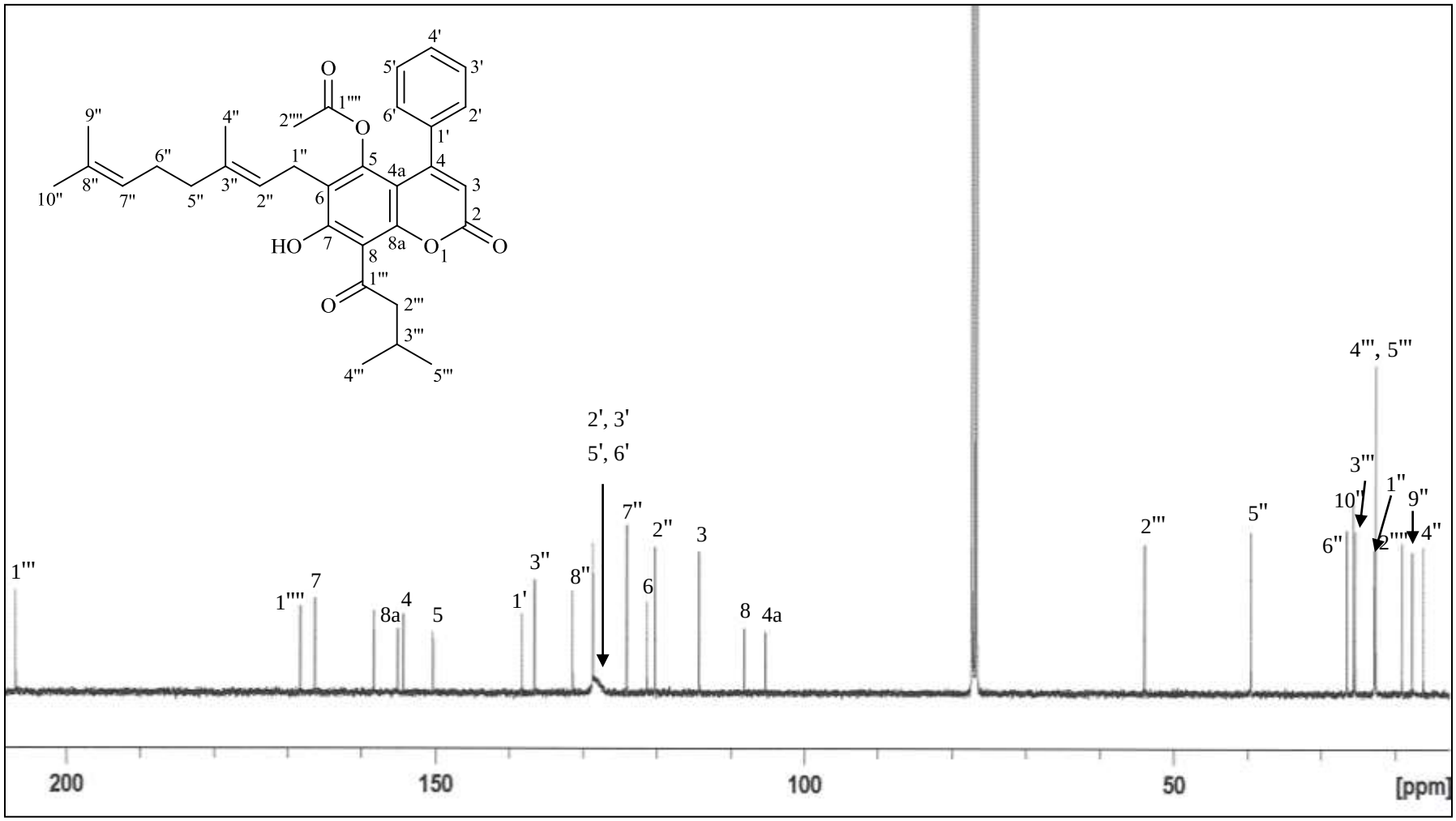


Figure 4.7: ^{13}C NMR Spectrum of Compound A4 **209**

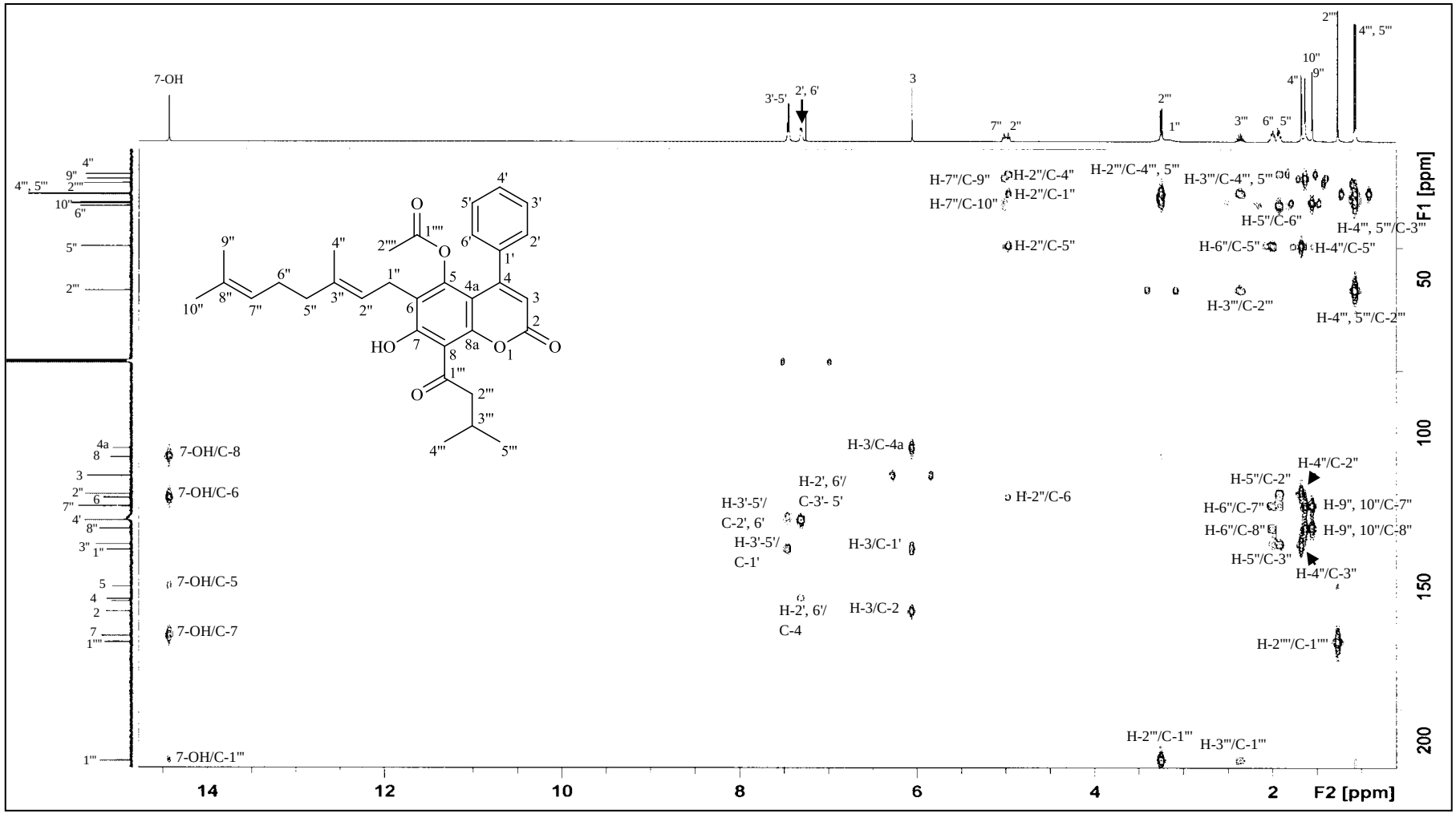
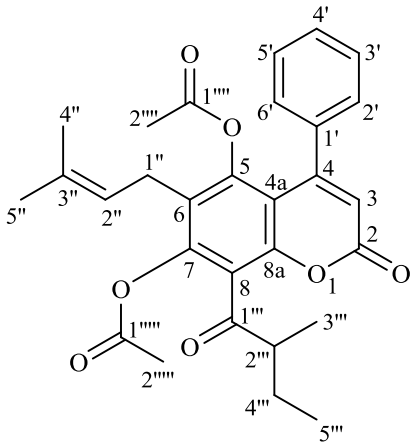


Figure 4.8: HMBC Spectrum of Compound A4 **209**

4.2.5 Compound A5: 8-(2-Methylbutanoyl)-6-(3-methylbut-2-enyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl diacetate 210



210

Compound A5 was acetylated from fraction F1 (hexane:ethyl acetate 95:5) of the hexane extract of *M. elegans* and isolated by PTLC and HPLC as a yellow oil. The HRESIMS spectrum exhibited a pseudomolecular $[M+Na]^+$ ion at *m/z* 513.1870 (calculated 513.1889), suggesting a molecular formula of $C_{29}H_{30}O_7$. The IR spectrum of compound A5 exhibited an absorption at υ_{max} 1732 cm^{-1}, which showed the presence of α-pyrone and ester groups. The UV spectrum (EtOH) of compound A5 showed peaks at 221, 295 and 330 nm, which showed resemblance with the UV absorbance of the parent compound F (226, 297, 331 nm), supporting the same skeletal structure.

An additional four carbons were apparent in the ^{13}C NMR spectrum of compound A5, as compared to the parent compound F, which has only twenty-five carbons. This indicated that both hydroxyl groups in the parent compound had been acetylated (5-OH and 7-OH). This observation was further supported by the absence of both hydroxyl peaks in the ^1H NMR spectrum of compound A5, as compared to the parent compound F. In addition, two methyl singlets were apparent in the ^1H NMR spectrum of compound A5; δ 1.23 (3H, *s*, H-2'''') and δ 2.24 (3H, *s*, H-2''''). The chemical shift of H-

2"'' was at higher field than the chemical shift of H-2"' due to the hydrogen bond of H-2"'' with the ketone group at C-1"'.

Based on a comparison of the spectral data between compound A5 and the parent compound F, the structure of compound A5 was determined to be 8-(2-methylbutanoyl)-6-(3-methylbut-2-enyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl diacetate **210**.

Table 4.8: ^{1}H NMR and ^{13}C NMR (in $CDCl_3$, 400 MHz) of Compound A5 **210**

Position	δ_H, *J* (Hz)	δ_C
2	-	158.1
3	6.19 (1H, *s*)	118.4
4	-	153.1
4a	-	111.8
5	-	147.2
6	-	126.0
7	-	150.4
8	-	122.2
8a	-	149.2
1'	-	137.4
2'	7.32 (1H, *m*, Ar)	127.8
3'	7.45 (3H, *m*, Ar)	128.8
4'		129.0
5'		128.8
6'	7.32 (1H, *m*, Ar)	127.8
1"	2.98 (1H, br*d*, *J* coupling obscured by peak H-2"')	25.6
2"	4.86 (1H, br*t*, *J* = 6.8)	120.3
3"	-	133.0
4"	1.61 (3H, *s*)	25.6
5"	1.61 (3H, *s*)	25.6
1"'	-	204.4
2"'	3.16 (1H, *sext*, *J* = 6.8)	48.9
3"'	1.23 (3H, brs)	18.0
4"'α	1.49 (1H, brs)	24.4
4"'β	1.90 (1H, brs)	
5"'	1.01 (3H, brs)	11.6
1""	-	168.4
2""	1.23 (3H, *s*)	19.1
1"""	-	168.6
2"""	2.24 (3H, *s*)	20.5

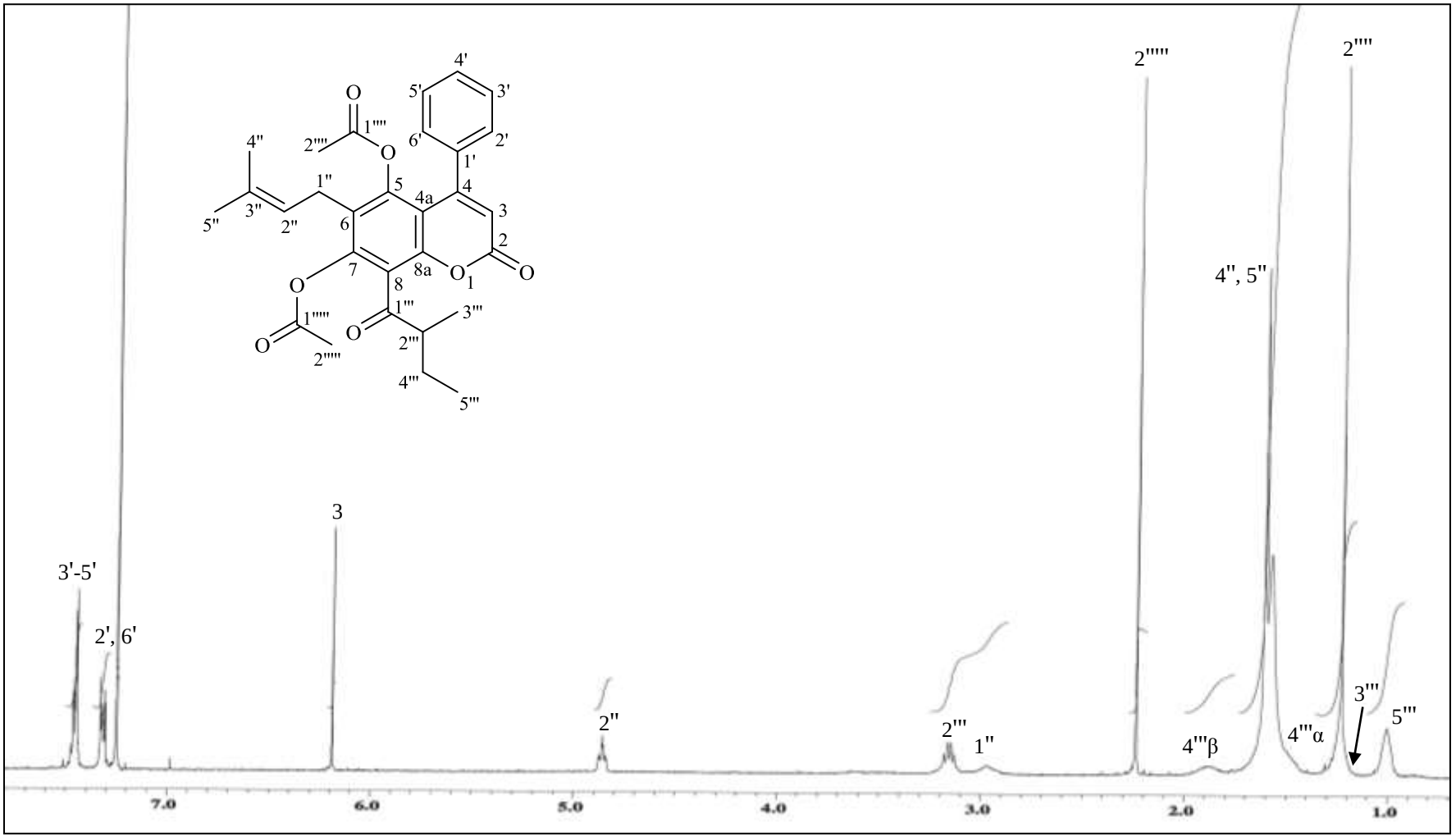
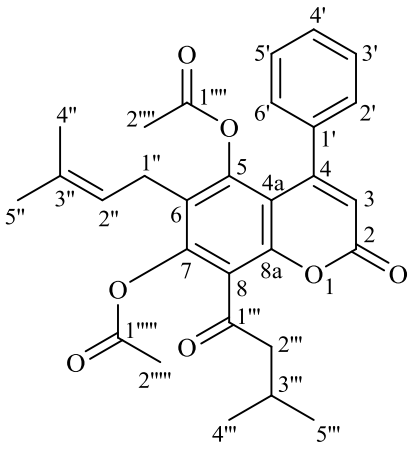


Figure 4.9: ^{1}H NMR Spectrum of Compound A5 **210**

4.2.6 Compound A6: 8-(3-Methylbutanoyl)-6-(3-methylbut-2-enyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl diacetate 211



211

Compound A6 was acetylated from the parent compound G, and then later separated, by PTLC and HPLC, as a white amorphous powder. The HRESIMS spectrum displayed pseudomolecular $[M+Na]^+$ ion at *m/z* 513.1847 (calculated 513.1889), signifying a molecular formula of $C_{29}H_{30}O_7$. The UV spectrum (EtOH) of compound A2 also supported the same skeletal structure as in compound G, by exhibiting peaks at 225, 297 and 330 nm. The IR spectrum of compound A2 showed noteworthy absorption at υ_{max} 1735 cm^{-1} (α,β-unsaturated lactone and ester groups). The IR peak for the hydroxyl group was absent in the IR spectrum, as compared to the parent compound G which displayed a hydroxyl group absorption at 3482 cm^{-1}. This suggested that acetylation had occurred at both the 5-OH and 7-OH hydroxyl groups.

The ^{13}C NMR spectrum showed twenty-nine carbon signals (six methyls, two methylenes, eight methines and thirteen quaternary carbons), indicating an additional four carbons as compared to the parent compound G which had twenty five carbons. The additional four carbon resonances in the ^{13}C NMR spectrum of compound A6 further supported the IR observations. Moreover, two additional methyl singlets were

observed in the ^{1}H NMR spectrum of compound A6; δ 1.25 (3H, *s*, H-2"") and δ 2.26 (3H, *s*, H-2""). On the other hand, the 5-OH and 7-OH signals were missing in the of compound A6, as compared to the ^{1}H NMR spectrum of the parent compound G. These observations indicated that the acetylation had taken place at positions C-5 and C-7 of the coumarin skeleton of compound A6.

Comparison of the spectral data between compound A6 and parent compound G indicated that this was an acetylated derivative of the parent compound G at positions C-5 and C-7, with the IUPAC name 8-(3-methylbutanoyl)-6-(3-methylbut-2-enyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl diacetate **211**.

Table 4.9: ^{1}H NMR and ^{13}C NMR (in $CDCl_3$, 400 MHz) of Compound A6 **211**

Position	δ_H, *J* (Hz)	δ_C
2	-	158.2
3	6.20 (1H, *s*)	118.3
4	-	153.1
4a	-	111.6
5	-	147.1
6	-	125.8
7	-	150.2
8	-	122.8
8a	-	148.8
1'	-	137.4
2'	7.32 (1H, *m*, Ar)	127.8
3'	7.45 (3H, *m*, Ar)	128.8
4'		129.0
5'		128.8
6'	7.32 (1H, *m*, Ar)	127.8
1"	2.93 (1H, br*d*, *J* coupling obscured by peak H-2"')	24.3
2"	4.86 (1H, br*t*, *J* = 6.8)	120.8
3"	-	133.1
4"	1.63 (3H, *s*)	17.9
5"	1.63 (3H, *s*)	25.6
1"'	-	200.2
2"'	2.92 (2H, *d*, *J*= 6.7)	53.2
3"'	2.33 (1H, *m*)	24.4
4"'	1.03 (6H, *d*, *J* = 6.7)	22.6
5"'		22.6
1""	-	168.4
2""	1.25 (3H, *s*)	19.1
1""'	-	168.6
2""'	2.26 (3H, *s*)	20.6

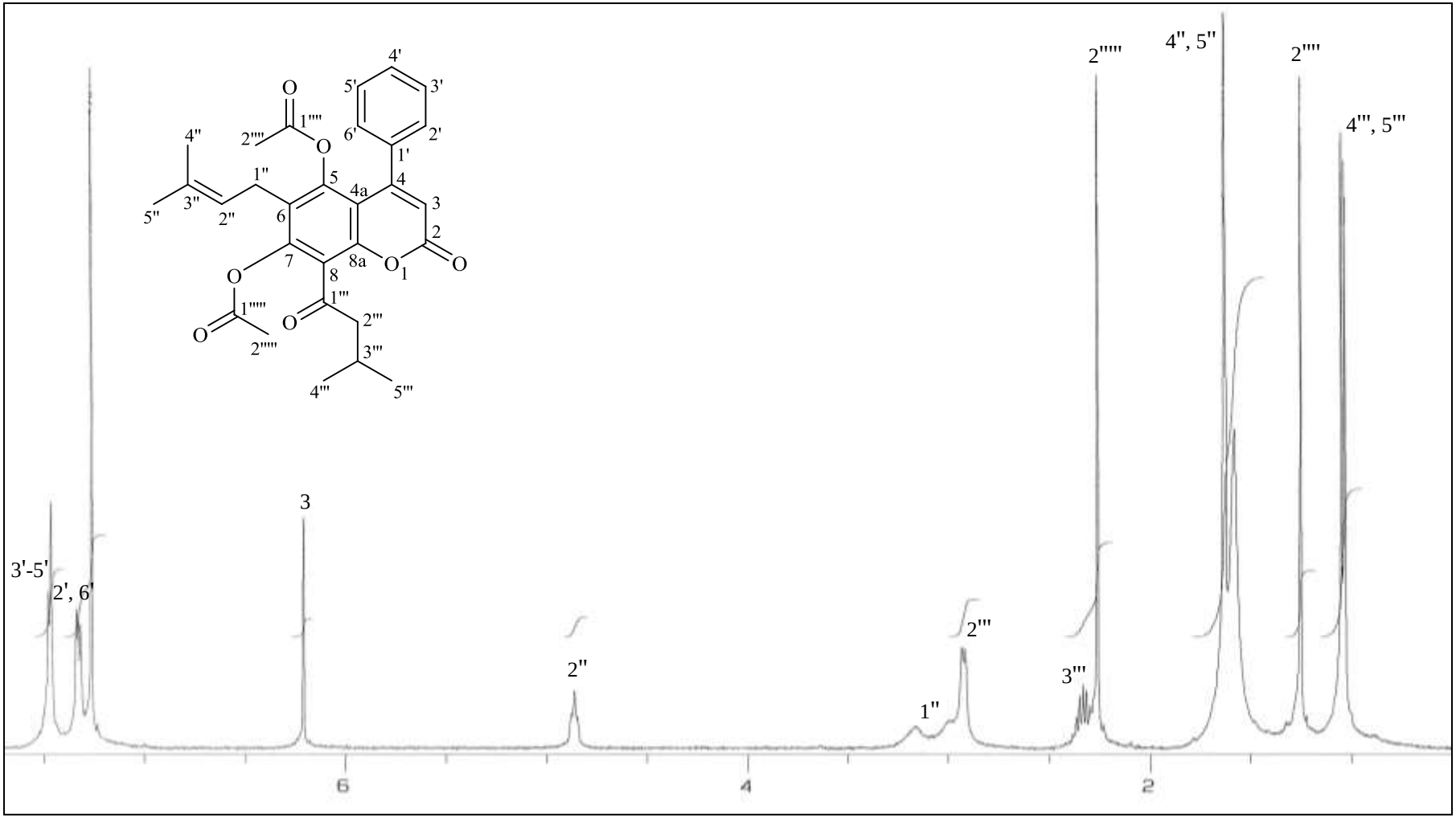


Figure 4.10: ^{1}H NMR Spectrum of Compound A6 **211**

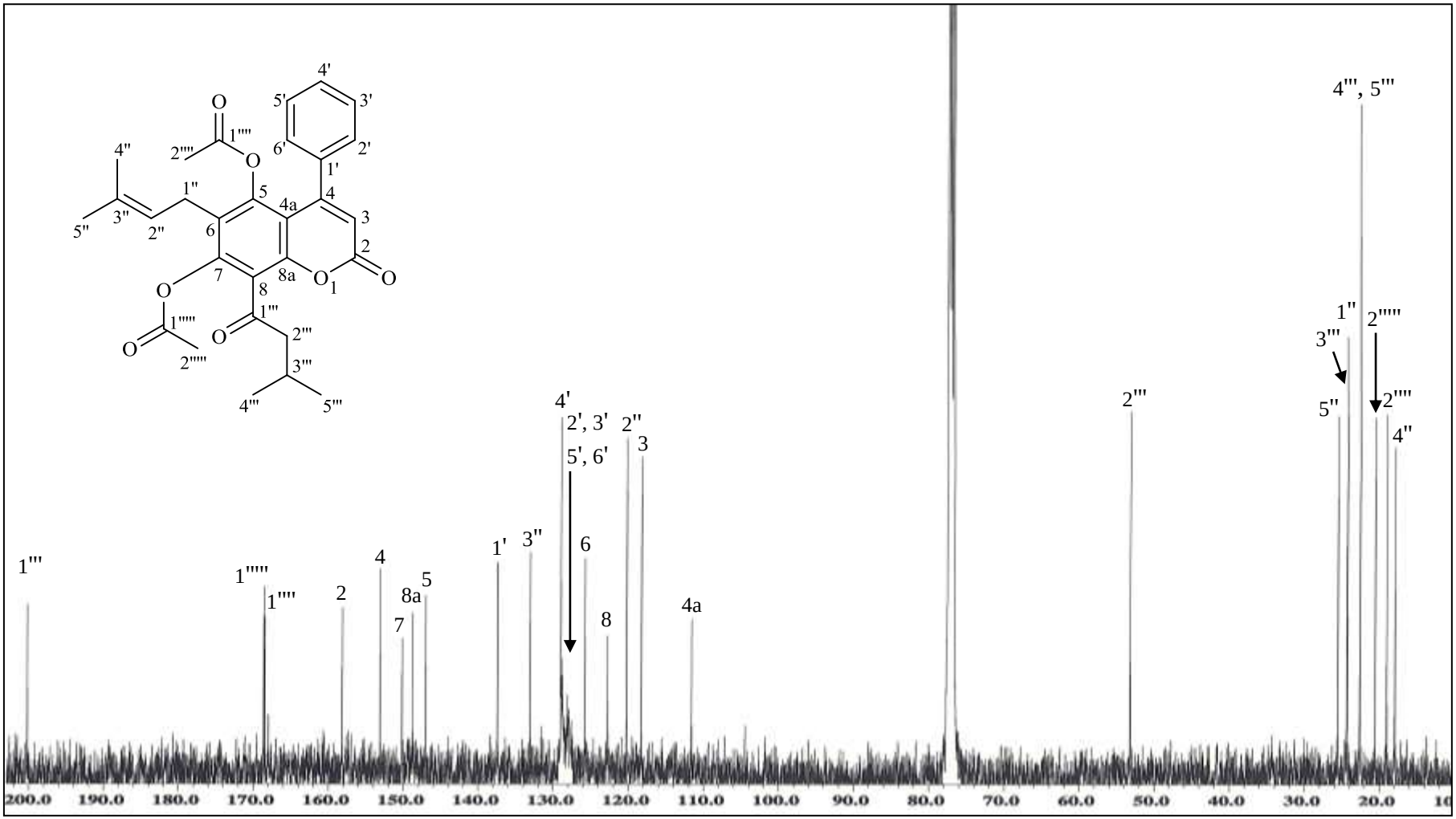
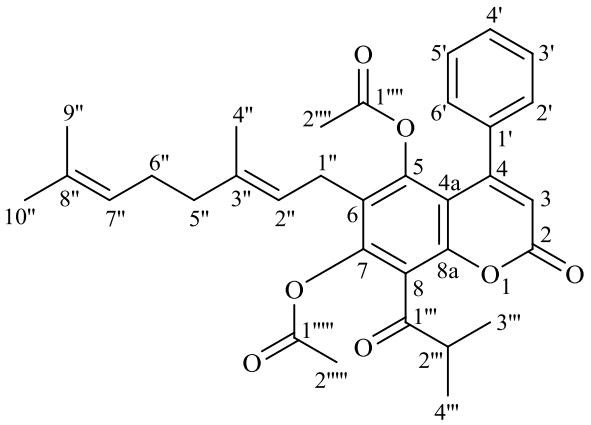


Figure 4.11: ^{13}C NMR Spectrum of Compound A6 **211**

4.2.7 Compound A7: 6-[(*E*)-3,7-Dimethylocta-2,6-dienyl]-8-(isobutyryl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl diacetate 212



212

Compound A7 was isolated as a white amorphous powder after being acetylated from compound H. The molecular formula of $C_{33}H_{36}O_7$ for compound A7 was established from the HRESIMS measurement which revealed a pseudomolecular $[M+Na]^+$ ion at *m/z* 567.2281 (calculated 567.2359). The IR spectrum showed absorptions at υ_{max} 1739 cm^{-1}, indicating the presence of α-pyrone and ester groups. The UV absorbance of compound A7 was similar to the parent compound H, with intense absorption bands at 225, 295 and 333 nm.

The ^{13}C NMR spectrum of compound A7 showed an addition of four carbons as compared to the parent compound H, with a total of thirty-three carbon signals (seven methyls, three methylenes, nine methines and fourteen quaternary carbons). In the ^1H NMR spectrum of compound A7, both 5-OH and 7-OH signals were missing as compared to the ^1H NMR spectrum of the parent compound. Instead, two methyl signals were apparent in the ^1H NMR spectrum of compound A7; δ 1.25 (3H, *s*, H-2'''') and δ 2.25 (3H, *s*, H-2''''). These indications suggested that acetylation had taken place at positions C-5 and C-7 of the parent compound H. Apart from the apparent differences in

the ^{1}H NMR spectra between compound A7 and the parent compound H, there were no other significant differences between the spectra of these two compounds.

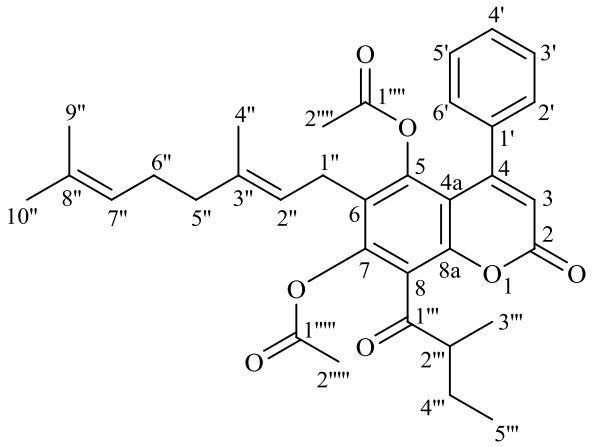
After analysis and comparison of the spectral data of compound A7 and the parent compound H, the structure of compound A7 was established as 6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-8-(isobutyryl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl diacetate **212**.

Table 4.10: ^{1}H NMR and ^{13}C NMR (in $CDCl_3$, 400 MHz) of Compound A7 **212**

Position	δ_H, *J* (Hz)	δ_C
2	-	158.2
3	6.20 (1H, *s*)	118.3
4	-	153.2
4a	-	111.7
5	-	147.1
6	-	126.0
7	-	150.1
8	-	122.1
8a	-	149.2
1'	-	137.4
2'	7.33 (1H, *m*, Ar)	128.0
3'	7.46 (3H, *m*, Ar)	128.8
4'		129.0
5'		128.8
6'	7.33 (1H, *m*, Ar)	128.0
1''	3.00 (1H, br*d*, *J* = 70.2)	24.3
2''	4.89 (1H, br*t*, *J* = 6.7)	120.1
3''	-	136.7
4''	1.62 (3H, *s*)	16.4
5''	1.93 (2H, *m*)	39.5
6''	1.98 (2H, *m*)	26.5
7''	5.03 (1H, br*t*, *J* = 6.7)	123.9
8''	-	131.7
9''	1.56 (3H, *s*)	17.8
10''	1.65 (3H, *s*)	25.8
1'''	-	204.8
2'''	3.31 (1H, *m*)	42.2
3'''	1.25 (6H, br*d*, *J* = 6.7)	18.1
4'''		18.1
1''''	-	168.4
2''''	1.25 (3H, *s*)	19.1
1'''''	-	168.6
2'''''	2.25 (3H, *s*)	20.6

Figure 4.12: ^{1}H NMR Spectrum of Compound A7 **212**

4.2.8 Compound A8: 6-[(*E*)-3,7-Dimethylocta-2,6-dienyl]-8-(2-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl diacetate 213



213

Fraction F1 (hexane:ethyl acetate 95:5) of the hexane extract of *M. elegans* was subjected to acetylation, and compound A8 was isolated from the product of this semi-synthesis process as a white amorphous powder. The HRESIMS spectrum exhibited pseudomolecular $[M+Na]^+$ ion at *m/z* 581.2495 (calculated 581.2515), signifying a molecular formula of $C_{34}H_{38}O_7$. The UV absorbance of compound A8 was similar to that of the parent compound I, with intense absorption bands at 223, 297 and 335 nm. The IR spectrum showed absorptions at υ_{max} 1732 cm^{-1}, indicating the presence of α-pyrone and ester groups.

Thirty-four carbon peaks were apparent in the ^{13}C NMR spectrum of compound A8 as seven methyls, four methylenes, nine methines and fourteen quaternary carbons. An addition of four carbons as compared to the parent compound I, which merely has 30 carbons indicated that acetylation had taken place at two different sites. The absence of the 5-OH and 7-OH peaks in the ^1H NMR spectrum of compound A8 further supported this indication. Moreover, two methyl singlets were observed in the ^1H NMR spectrum of compound A8 at δ 1.23 (3H, *s*, H-2'''') and at δ 2.24 (3H, *s*, H-2'''').

These observations led to the conclusion that compound A8 was a di-acetylated product of the parent compound I, with the IUPAC name of 6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-8-(2-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl diacetate **213**.

Table 4.11: ^{1}H NMR and ^{13}C NMR (in $CDCl_3$, 400 MHz) of Compound A8 **213**

Position	δ_H, *J* (Hz)	δ_C
2	-	158.0
3	6.19 (1H, *s*)	118.3
4	-	153.0
4a	-	111.7
5	-	147.1
6	-	126.0
7	-	150.3
8	-	122.2
8a	-	149.2
1'	-	137.4
2'	7.32 (1H, *m*, Ar)	127.8
3'	7.45 (3H, *m*, Ar)	128.3
4'		128.9
5'		128.3
6'	7.32 (1H, *m*, Ar)	127.8
1"	2.99 (1H, br*d*, *J* coupling obscured by H-2"')	22.9
2"	4.87 (1H, br*t*, *J* = 6.84)	120.1
3"	-	136.6
4"	1.61 (3H, *s*)	16.3
5"	1.93 (2H, *m*)	39.4
6"	1.98 (2H, *m*)	26.4
7"	5.02 (1H, br*t*, *J* = 6.72)	123.9
8"	-	131.6
9"	1.55 (3H, *s*)	17.7
10"	1.64 (3H, *s*)	25.6
1"'	-	204.3
2"'	3.18 (1H, *m*)	48.8
3"'	1.23 (3H, br*s*)	16.2
4"'α	1.49 (1H, *m*)	25.4
4"'β	1.89 (1H, *m*)	
5"'	1.01 (3H, br*s*)	11.5
1""	-	168.2
2""	1.23 (3H, *s*)	19.0
1""'	-	168.4
2""'	2.24 (3H, *s*)	20.4

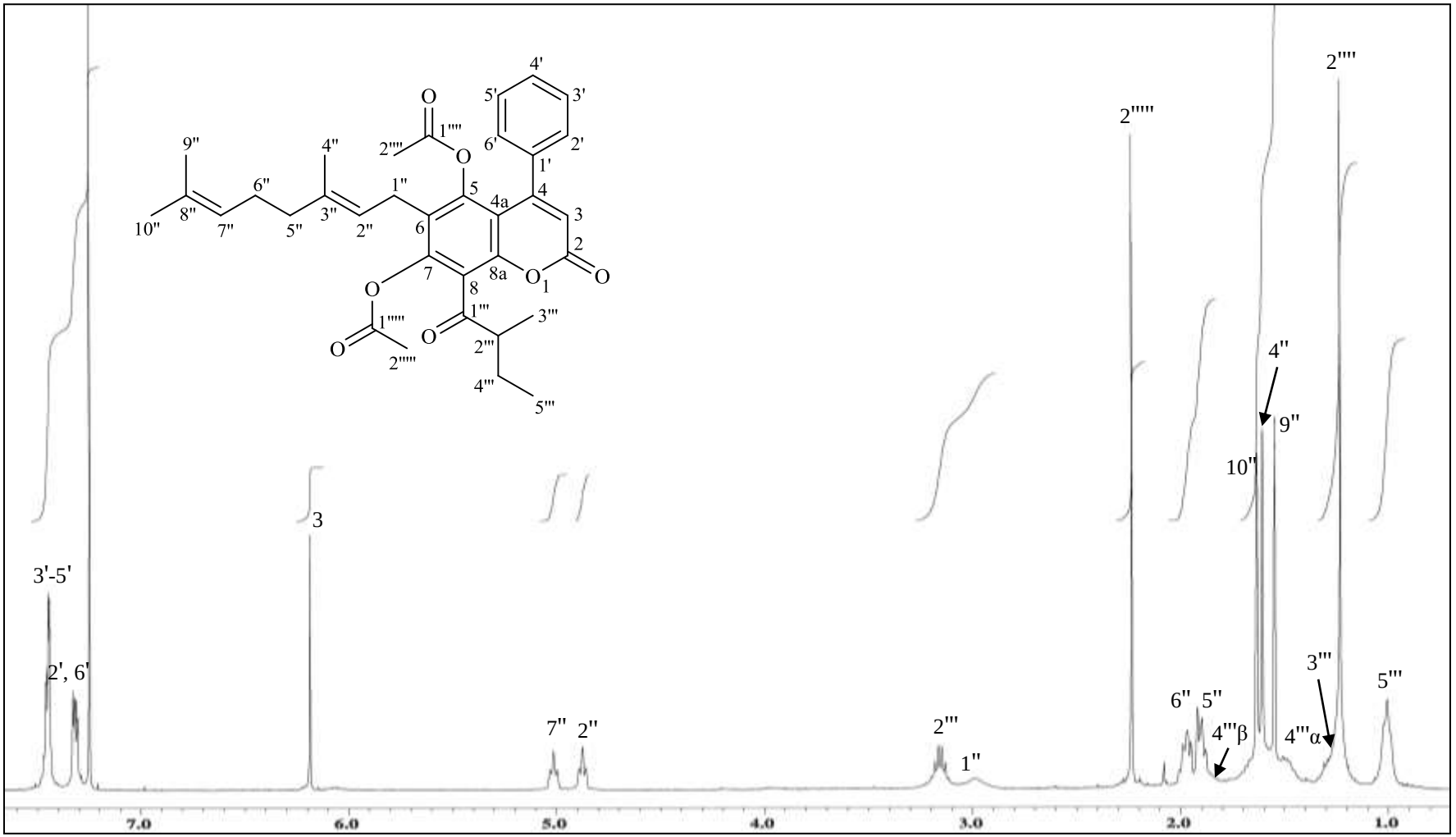


Figure 4.13: ^{1}H NMR Spectrum of Compound A8 **213**

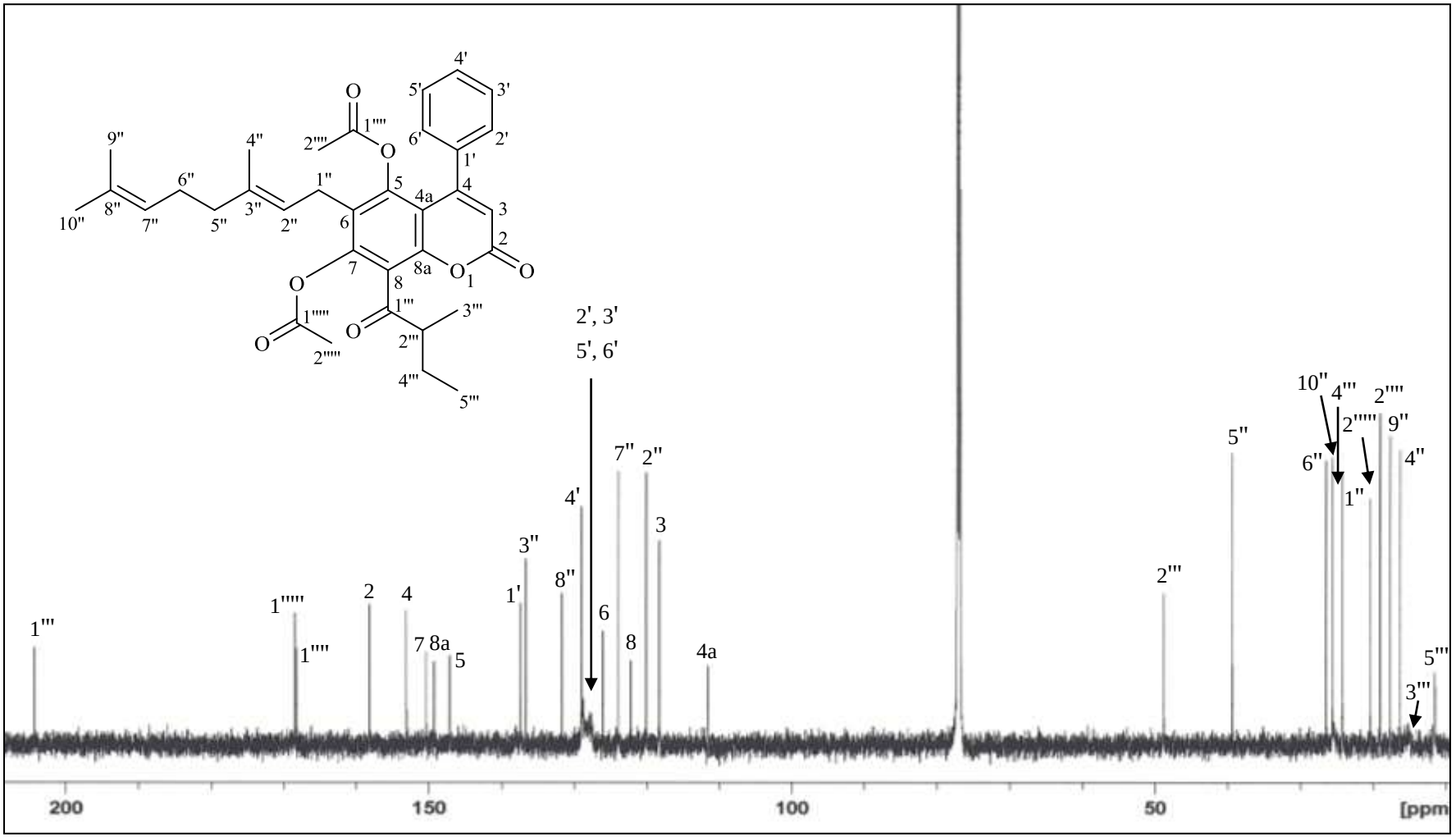
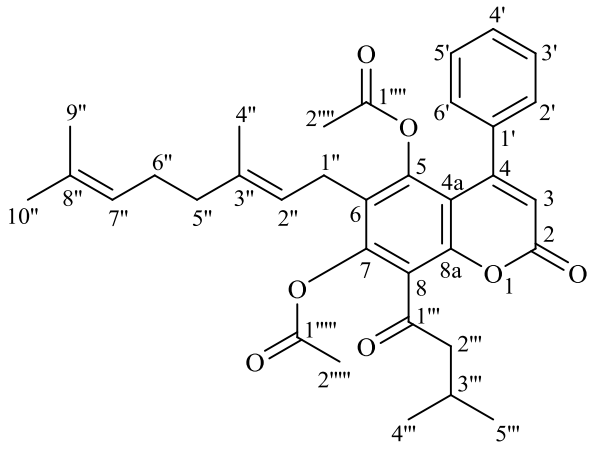


Figure 4.14: ^{13}C NMR Spectrum of Compound A8 **213**

4.2.9 Compound A9: 6-[(*E*)-3,7-Dimethylocta-2,6-dienyl]-8-(3-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl diacetate 214



214

Compound A9 was acetylated from the parent compound J and obtained through PTLC and HPLC separations as a white amorphous powder. The molecular formula of $C_{34}H_{38}O_7$ was established for compound A9 from the HRESIMS spectrum, which exhibited a pseudomolecular $[M+Na]^+$ ion at *m/z* 581.2503 (calculated 581.2515). The UV absorbance of compound A9 was reminiscent with the parent compound J, with absorbance peaks at 225, 295 and 333 nm, thus supporting the same skeletal structure of the parent compound J. In the IR spectrum of compound A9, as compared to the IR spectrum of parent compound J, the absence of hydroxyl group signal led to the suggestion that the 5-OH and 7-OH hydroxyl groups were acetylated.

These observations from the IR spectrum of compound A9 were further supported by the absence of 5-OH and 7-OH peak signals in the ^{1}H NMR spectrum of compound A9, as compared to the parent compound J. In addition, two methyl singlets were observed in the ^{1}H NMR spectrum of compound A9; δ 1.24 (3H, *s*, H-2'''') and δ 2.25 (3H, *s*, H-2''''). This observation further supported the inference that acetylation had occurred at the 5-OH and the 7-OH. Moreover, the observations from ^{1}H NMR and IR spectra of

compound A9 was further reinforced by the ^{13}C NMR spectrum of compound A9, which displayed thirty-four carbon resonances (seven methyls, four methylenes, nine methines and fourteen quaternary carbons) as compared to the parent compound J which had thirty carbons. Apart from the apparent differences in the ^{1}H and ^{13}C NMR spectra of compound A9, the other substituents of the coumarin skeleton (4-phenyl, geranyl and 3-methylbutanoyl) remained unchanged.

Detailed analysis of the 1D and 2D NMR data of compound A9 led to the complete NMR assignment of compound A9, which was established as 6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-8-(3-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl diacetate **214**.

Table 4.12: ^{1}H NMR and ^{13}C NMR (in $CDCl_3$, 400 MHz) of Compound A9 **214**

Position	δ_H, *J* (Hz)	δ_C
2	-	158.2
3	6.19 (1H, *s*)	118.2
4	-	153.2
4a	-	111.6
5	-	147.1
6	-	125.9
7	-	150.2
8	-	122.8
8a	-	148.8
1'	-	137.4
2'	7.31 (1H, *m*, Ar)	128.0
3'	7.45 (3H, *m*, Ar)	128.8
4'		129.0
5'		128.8
6'	7.31 (1H, *m*, Ar)	128.0
1"	3.00 (1H, br*d*, *J* = 65.4)	24.2
2"	4.86 (1H, br*t*, *J* = 6.8)	120.1
3"	-	136.8
4"	1.62 (3H, *s*)	16.4
5"	1.93 (2H, *m*)	39.5
6"	1.98 (2H, *m*)	26.5
7"	5.01 (1H, br*t*, *J* = 6.8)	123.9
8"	-	131.7
9"	1.55 (3H, *s*)	17.7
10"	1.64 (3H, *s*)	25.7
1'''	-	200.2
2'''	2.90 (2H, *d*, *J* = 6.4)	53.2
3'''	2.32 (1H, *m*)	24.3
4'''	1.02 (6H, *d*, *J* = 6.4)	22.6
5'''		22.6
1''''	-	168.4
2''''	1.24 (3H, *s*)	19.1
1'''''	-	168.6
2'''''	2.25 (3H, *s*)	20.6

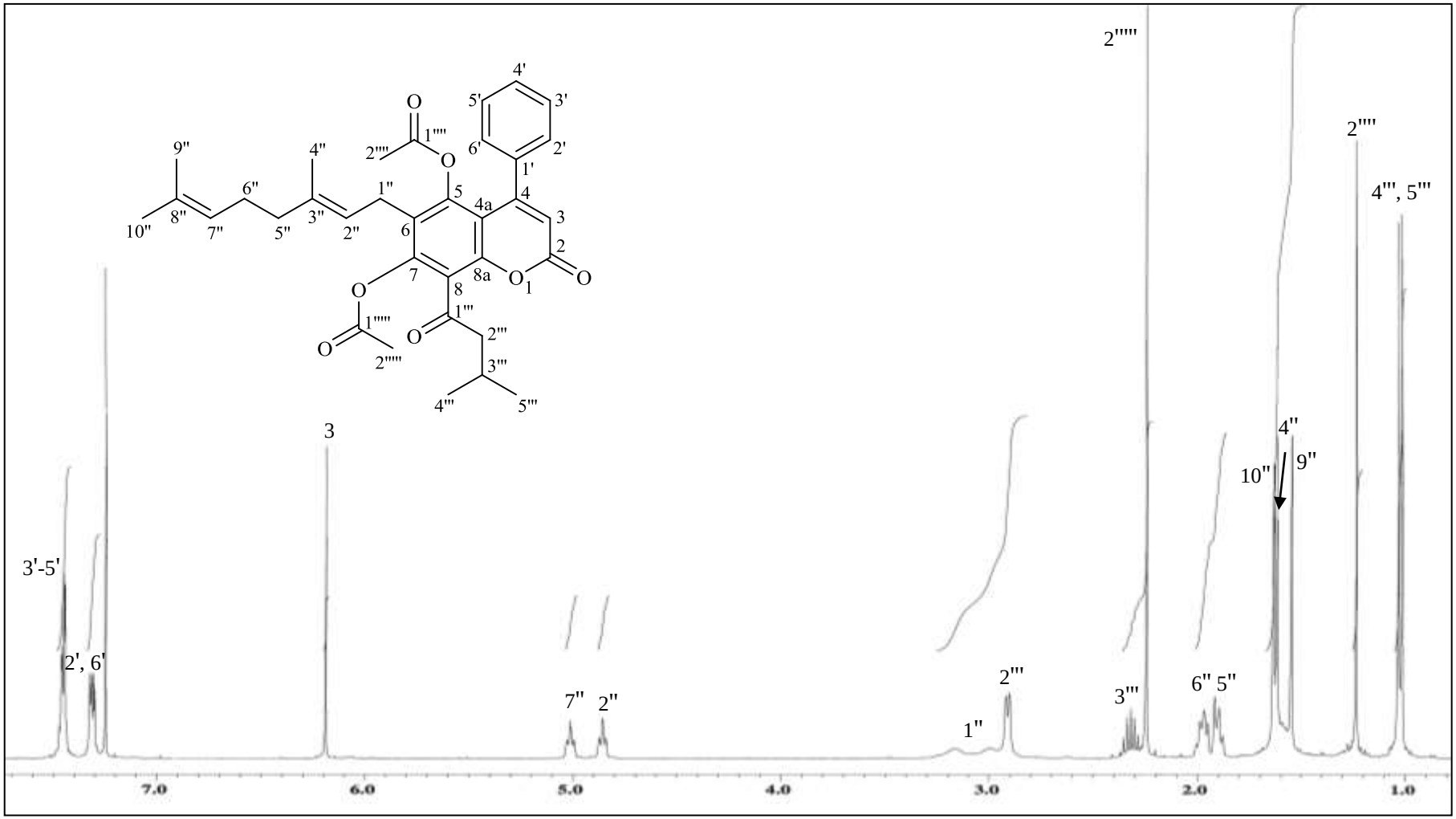


Figure 4.15: ^{1}H NMR Spectrum of Compound A9 **214**

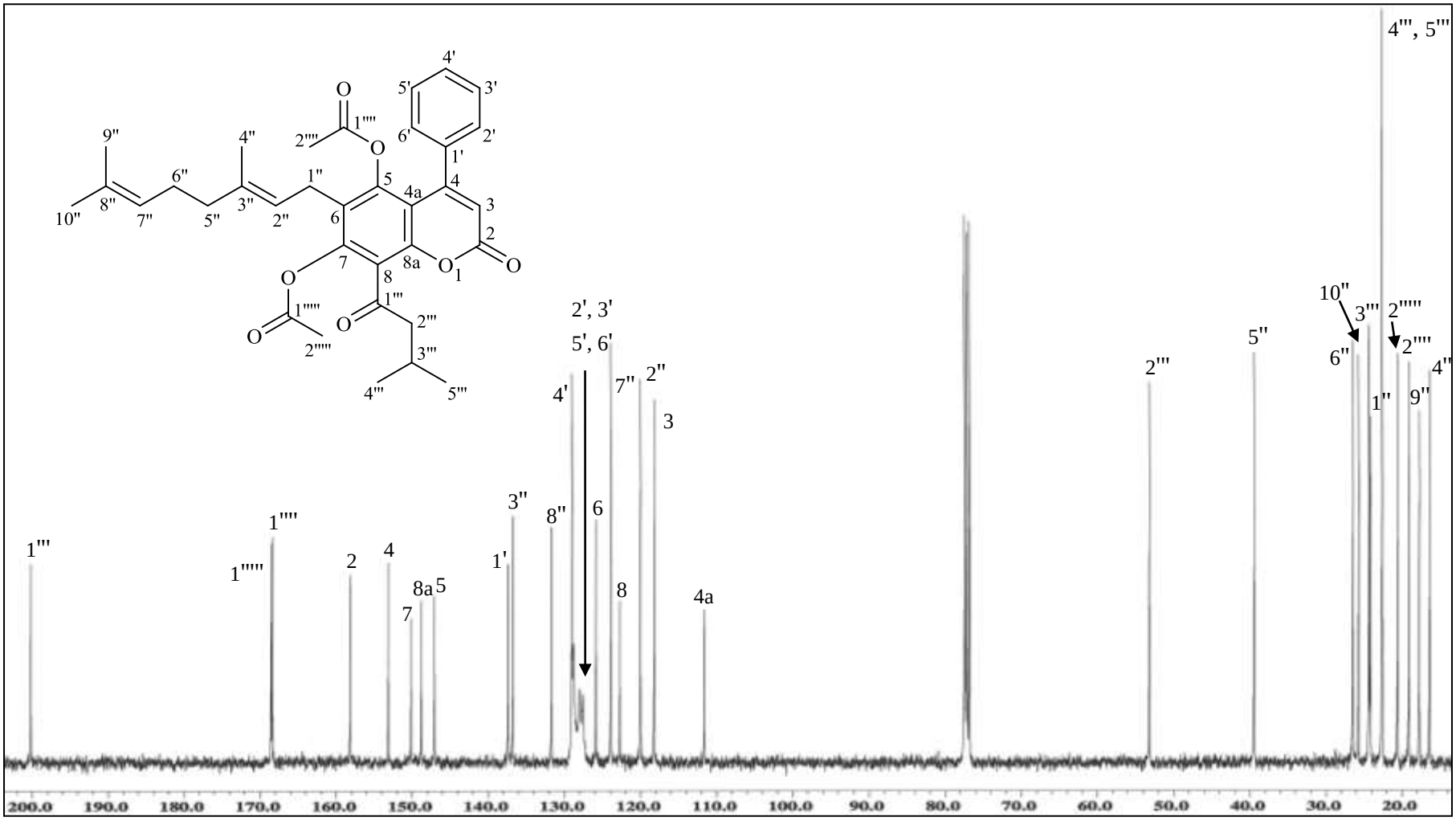


Figure 4.16: ^{13}C NMR Spectrum of Compound A9 **214**

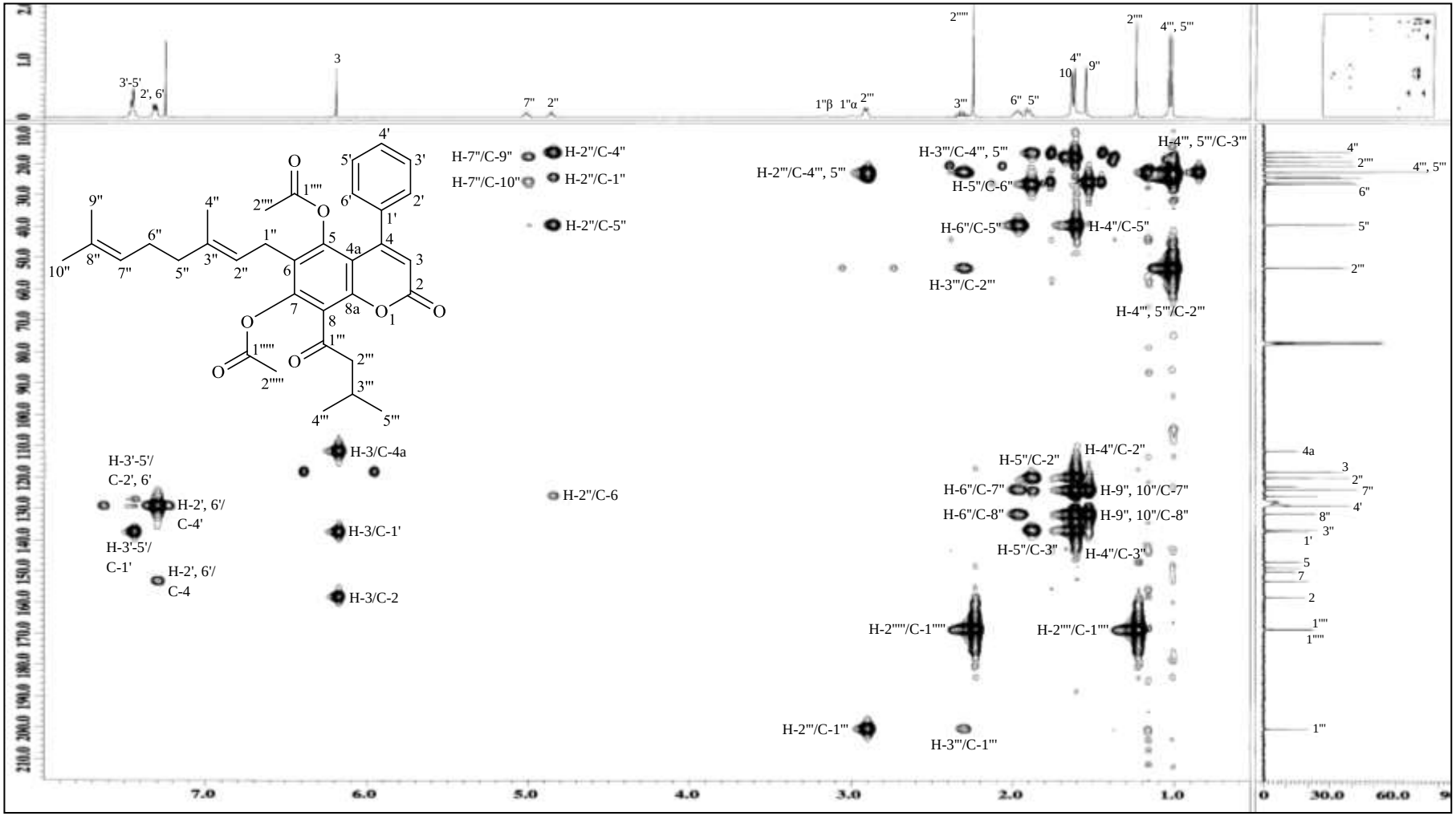
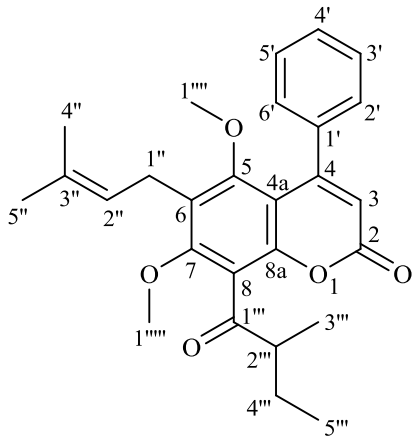


Figure 4.17: HMBC Spectrum of Compound A9 **214**

4.2.10 Compound M1: 5,7-Dimethoxy-8-(2-methylbutanoyl)-6-(3-methylbut-2-enyl)-4-phenyl-2*H*-chromen-2-one 215



215

Compound M1 was methylated from fraction F1 (hexane:ethyl acetate 95:5) of the hexane extract of *M. elegans*. Compound M1 was isolated through PTLC and HPLC as a colourless oil. The HRESIMS spectrum exhibited pseudomolecular $[M+Na]^+$ at *m/z* 457.1875 (calculated 457.1991), suggesting a molecular formula of $C_{27}H_{30}O_5$. The UV spectrum (EtOH) of compound M1 showed peaks at 225, 297 and 331nm, which showed a resemblance with the UV absorbance of the parent compound F (226, 297, 331 nm), supporting the same skeletal structure. In the IR spectrum of compound M1, the hydroxyl group peaks were not observed, suggesting that methylation had taken place on both of the hydroxyl groups.

The ^{13}C NMR spectrum of compound M1 showed six methyl signals, whereas the parent compound F only displayed four methyl signals. In the ^1H NMR spectrum of compound M1, proton signals for the 5-OH and 7-OH, which were apparent in the ^1H NMR spectrum of the parent compound F at δ 5.94 and δ 14.57, respectively, were missing. On the contrary, two methoxy signals were apparent in the ^1H NMR spectrum of compound M1 at δ 2.97 (3H, *s*, H_3-1'''') and δ 3.79 (3H, *s*, H_3-1''''''), which were

absent in the spectrum of the parent compound F. These observations from the spectral data led to the conclusion that compound M1 was methylated at positions C-5 and C-7.

The observed data of compound M1 suggested that this was a di-methylated derivative of the parent compound F at positions C-5 and C-7, with the IUPAC name 5,7-dimethoxy-8-(2-methylbutanoyl)-6-(3-methylbut-2-enyl)-4-phenyl-2*H*-chromen-2-one **215**.

Table 4.13: ^{1}H NMR and ^{13}C NMR (in $CDCl_3$, 400 MHz) of Compound M1 **215**

Position	δ_H, *J* (Hz)	δ_C
2	-	159.3
3	6.17 (1H, *s*)	116.2
4	-	154.4
4a	-	109.8
5	-	157.4
6	-	127.0
7	-	159.0
8	-	121.8
8a	-	150.5
1'	-	138.3
2'	7.37 (1H, *m*, Ar)	127.8
3'	7.41 (3H, *m*, Ar)	128.3
4'		128.7
5'		128.3
6'	7.37 (1H, *m*, Ar)	127.8
1"	3.30 (1H, *d*, *J* = 6.4)	21.7
2"	5.09 (1H, br*t*, *J* = 6.4)	120.8
3"	-	133.0
4"	1.71 (3H, *s*)	17.9
5"	1.67 (3H, *s*)	25.6
1'"	-	206.2
2'"	3.01 (1H, *m*)	49.0
3'"	1.24 (3H, *d*, *J* = 7.0)	16.3
4'"α	1.49 (1H, *m*)	27.0
4'"β	1.90 (1H, *m*)	
5'"	1.02 (3H, *t*, *J* = 7.3)	11.8
1""	2.97 (1H, *s*)	62.2
1"""	3.79 (3H, *s*)	63.6

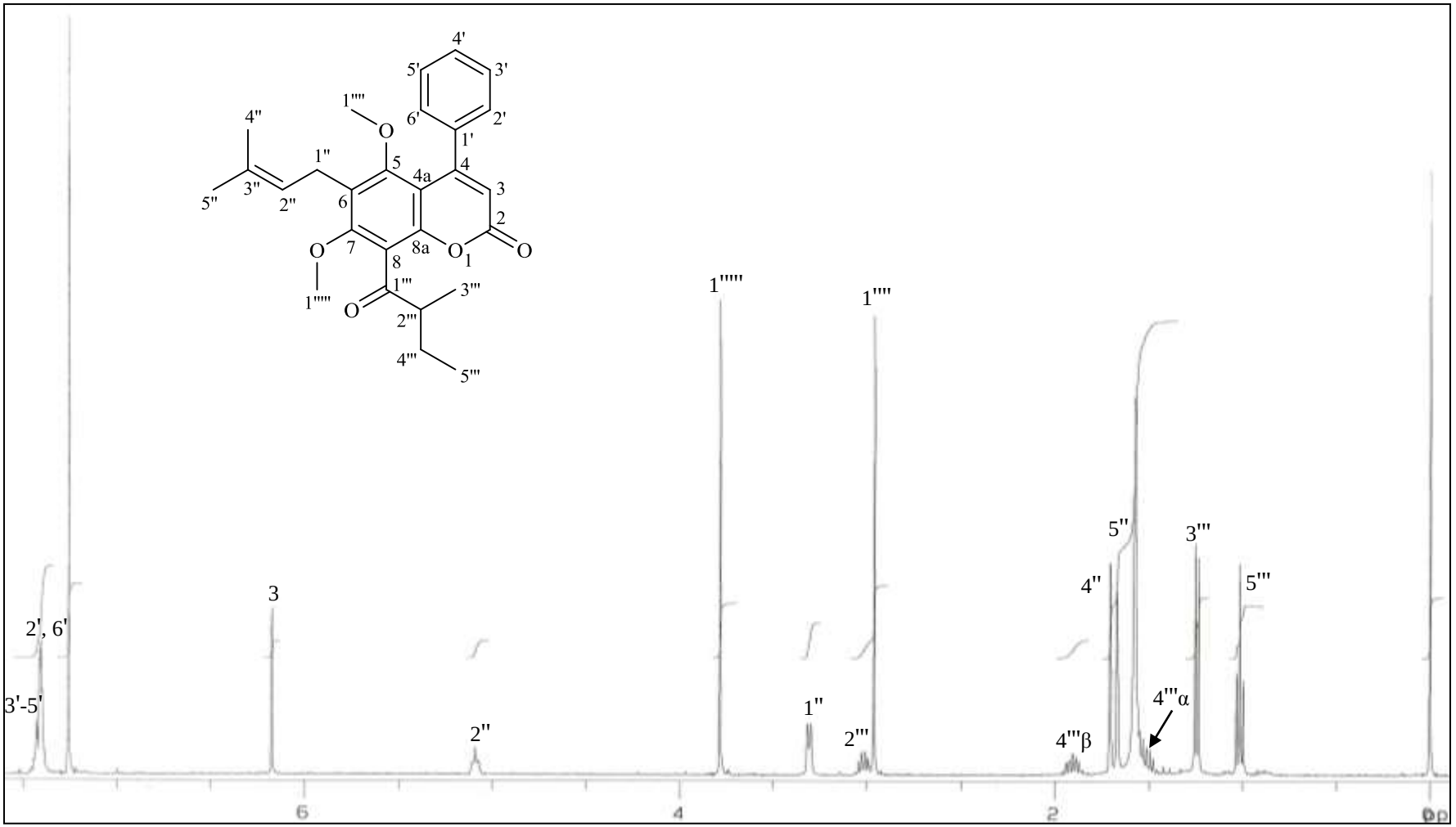
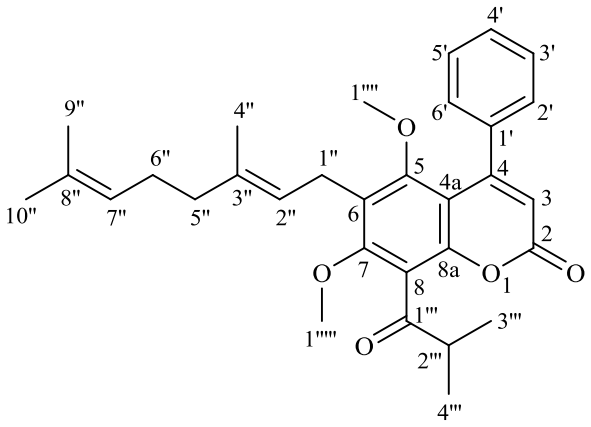


Figure 4.18: ^{1}H NMR Spectrum of Compound M1 **215**

4.2.11 Compound M2: 5,7-Dimethoxy-6-[(*E*)-(3,7-dimethylocta-2,6-dienyl]-8-isobutyryl-4-phenyl-2*H*-chromen-2-one 216



216

Fraction F1 (hexane:ethyl acetate 95:5) of the hexane extract of *M. elegans* was subjected to methylation, and compound M2 was isolated through PTLC and HPLC separations from the product of this semi-synthetic process as a white amorphous powder. The molecular formula of $C_{31}H_{36}O_5$ was obtained from the HRESIMS spectrum of compound M2, which exhibited a pseudomolecular $[M+Na]^+$ ion at *m/z* 511.2587 (calculated 511.2460). The UV absorbance of compound M2 was similar with the parent compound H, to that of intense absorption peaks at 223, 295 and 335 nm. The IR spectrum showed an absorption at υ_{max} 1735cm^{-1} indicating the presence of α-pyrone group.

The ^{13}C NMR spectrum of compound M2 displayed thirty-one carbon signals (seven methyls, three methylenes, nine methines and twelve quaternary carbons), as compared to the parent compound H which has twenty-nine carbons signals. This observation suggests that the hydroxyl groups at 5-OH and 7-OH were methylated. In addition, the absence of the 5-OH and 7-OH resonances in the ^{1}H NMR spectrum of compound M2 further supported this observation. Moreover, two methoxy singlets were observed in

the ^{1}H NMR spectrum of compound M2 at δ 2.96 (3H, *s*, H_3-1"") and at δ 3.78 (3H, *s*, H_3-1""), which further suggested that C-5 and C-7 of compound M2 had been methylated. Apart from the marked differences between the ^{1}H NMR spectra of compound M2 and the parent compound H, signals for the other substituents of the coumarin skeleton showed similarities (4-phenyl, geranyl and butanoyl groups).

These observations led to the conclusion that compound M2 was indeed a di-methylated product of the parent compound H, for which the IUPAC name of 5,7-dimethoxy-6-[(*E*)-(3,7-dimethylocta-2,6-dienyl]-8-isobutyryl-4-phenyl-2*H*-chromen-2-one was assigned **216**.

Table 4.14: ^{1}H NMR and ^{13}C NMR (in $CDCl_3$, 400 MHz) of Compound M2 **216**

Position	δ_H, *J* (Hz)	δ_C
2	-	159.3
3	6.16 (1H, *s*)	116.2
4	-	154.4
4a	-	109.8
5	-	157.5
6	-	126.9
7	-	158.8
8	-	122.2
8a	-	150.3
1'	-	129.9
2'	7.37 (1H, *m*, Ar)	127.5
3'	7.41 (3H, *m*, Ar)	128.0
4'		128.8
5'		128.0
6'	7.37 (1H, *m*, Ar)	127.5
1''	3.31 (2H, *d*, *J* = 6.4)	23.2
2''	5.08 (1H, br*t*, *J* = 6.4)	122.8
3''	-	136.1
4''	1.70 (3H, *s*)	16.3
5''	1.97 (2H, *m*)	39.5
6''	2.03 (2H, *m*)	26.7
7''	5.02 (1H, br*t*, *J* = 6.8)	124.1
8''	-	131.6
9''	1.56 (3H, *s*)	17.7
10''	1.62 (3H, *s*)	25.9
1'''	-	202.9
2'''	3.17 (1H, *m*)	42.2
3'''	1.26 (6H, *d*, *J* = 6.7)	18.0
4'''		18.0
1''''	2.96 (3H, *s*)	62.3
1'''''	3.78 (3H, *s*)	63.4

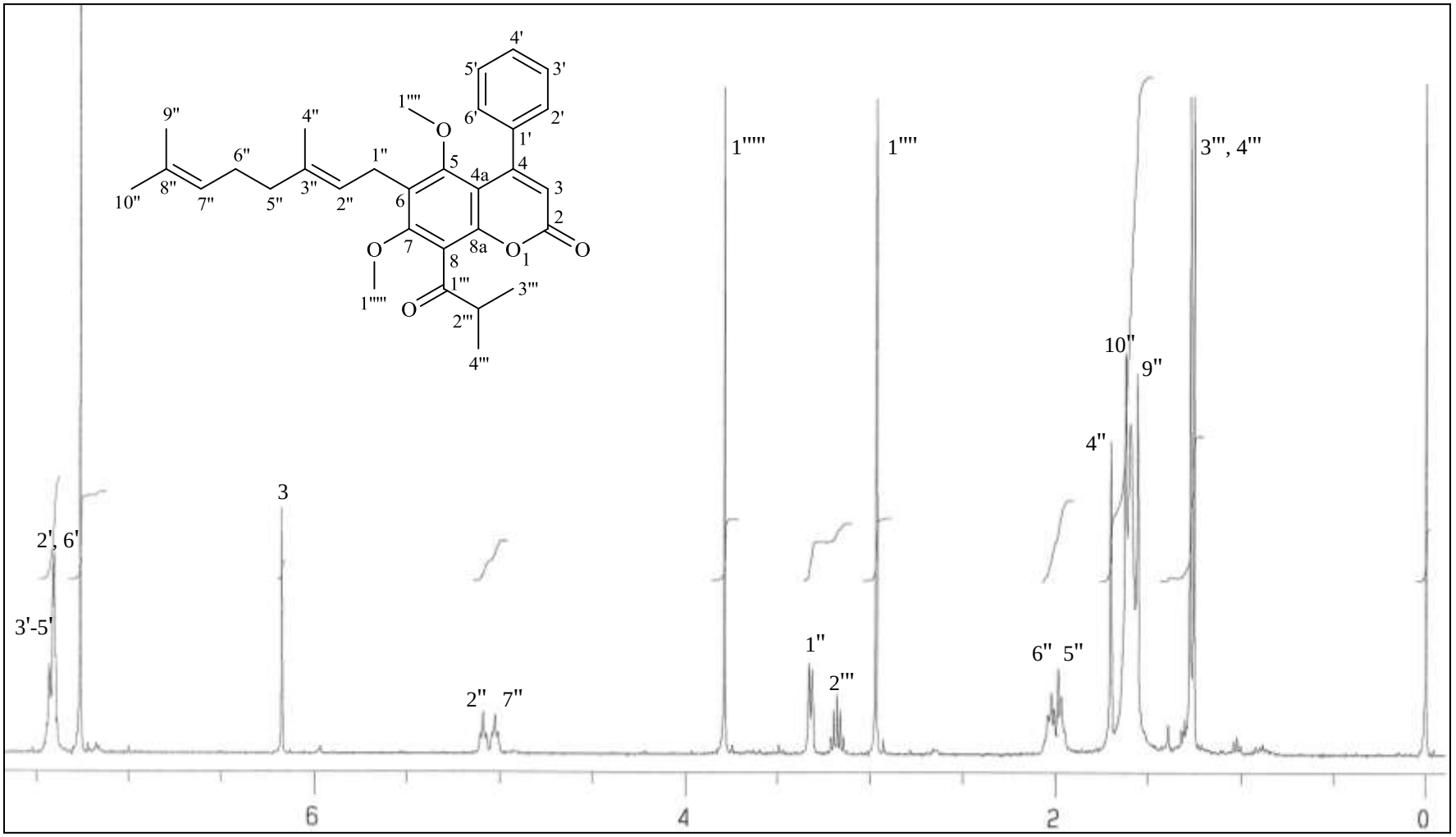
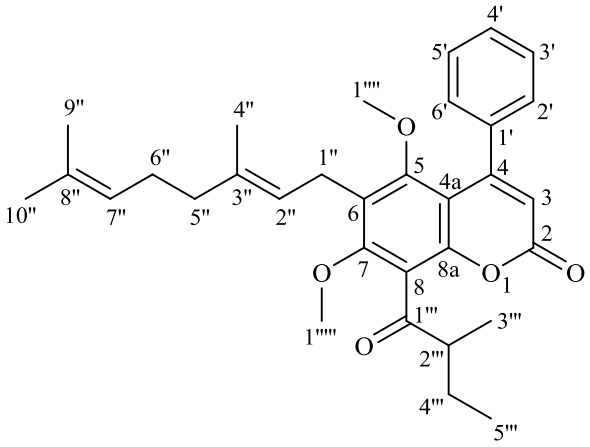


Figure 4.19: ^{1}H NMR Spectrum of Compound M2 **216**

4.2.12 Compound M3: 5,7-Dimethoxy-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-8-(2-methylbutanoyl)-4-phenyl-2*H*-chromen-2-one 217



217

Compound M3 was methylated from the parent compound I and was isolated through PTLC and HPLC separations as a white amorphous powder. The HRESIMS spectrum revealed a pseudomolecular $[M+Na]^+$ ion at *m/z* 525.2533 (calculated 525.2617), suggesting a molecular formula of $C_{32}H_{38}O_5$. The UV spectrum showed maximum absorption bands at 225, 297 and 332 nm, which were similar to the parent compound I (226, 296 and 333 nm), indicating the same skeletal structure. In the IR spectrum of compound M3, the hydroxyl group absorption were not observed, suggesting that methylation had taken place on both of the hydroxyl groups.

An additional two carbons were apparent in the ^{13}C NMR spectrum of compound M3, as compared to the parent compound I. This indicated that both of the hydroxyl groups in the parent compound I (at 5-OH and 7-OH) had been methylated. This observation was further supported by the absence of both hydroxyl peaks in the ^1H NMR spectrum of compound M3, as compared to parent compound I. In addition, two methoxy singlets were apparent in the ^1H NMR spectrum of compound M3 at δ 2.97 (3H, *s*, H_3-1'''') and δ 3.78 (3H, *s*, H_3-1''''). The chemical shift of H-1'''' was more down field than the

chemical shift of H-1"" due to the hydrogen bond of H-2""" with the ketone group at C-1"'.

With the comparison of spectral data between compound M3 and the parent compound I, the structure of compound M3 was determined as 5,7-dimethoxy-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-8-(2-methylbutanoyl)-4-phenyl-2*H*-chromen-2-one **217**.

Table 4.15: ^{1}H NMR and ^{13}C NMR (in $CDCl_3$, 400 MHz) of Compound M3 **217**

Position	δ_H, *J* (Hz)	δ_C
2	-	159.3
3	6.16 (1H, *s*)	116.2
4	-	154.4
4a	-	109.9
5	-	157.6
6	-	127.0
7	-	159.0
8	-	121.9
8a	-	150.5
1'	-	138.1
2'	7.37 (1H, *m*, Ar)	127.6
3'	7.41 (3H, *m*, Ar)	128.0
4'		128.8
5'		128.0
6'	7.37 (1H, *m*, Ar)	127.6
1"	3.20 (1H, *d*, *J* = 6.4)	23.1
2"	5.07 (1H, br*t*, *J* = 6.4)	122.7
3"	-	136.1
4"	1.69 (3H, *s*)	16.3
5"	1.95 (2H, *m*)	39.6
6"	2.01 (2H, *m*)	26.5
7"	5.01 (1H, br*t*, *J* = 6.8)	124.1
8"	-	131.6
9"	1.54 (3H, *s*)	17.7
10"	1.61 (3H, *s*)	25.7
1'"	-	206.3
2'"	3.00 (1H, *m*)	49.3
3'"	1.22 (3H, *d*, *J* = 6.8)	14.9
4'"α	1.49 (1H, *m*)	25.1
4'"β	1.90 (1H, *m*)	
5'"	1.00 (3H, *t*, *J* = 7.4)	11.7
1""	2.95 (3H, *s*)	62.3
1"""	3.78 (3H, *s*)	63.6

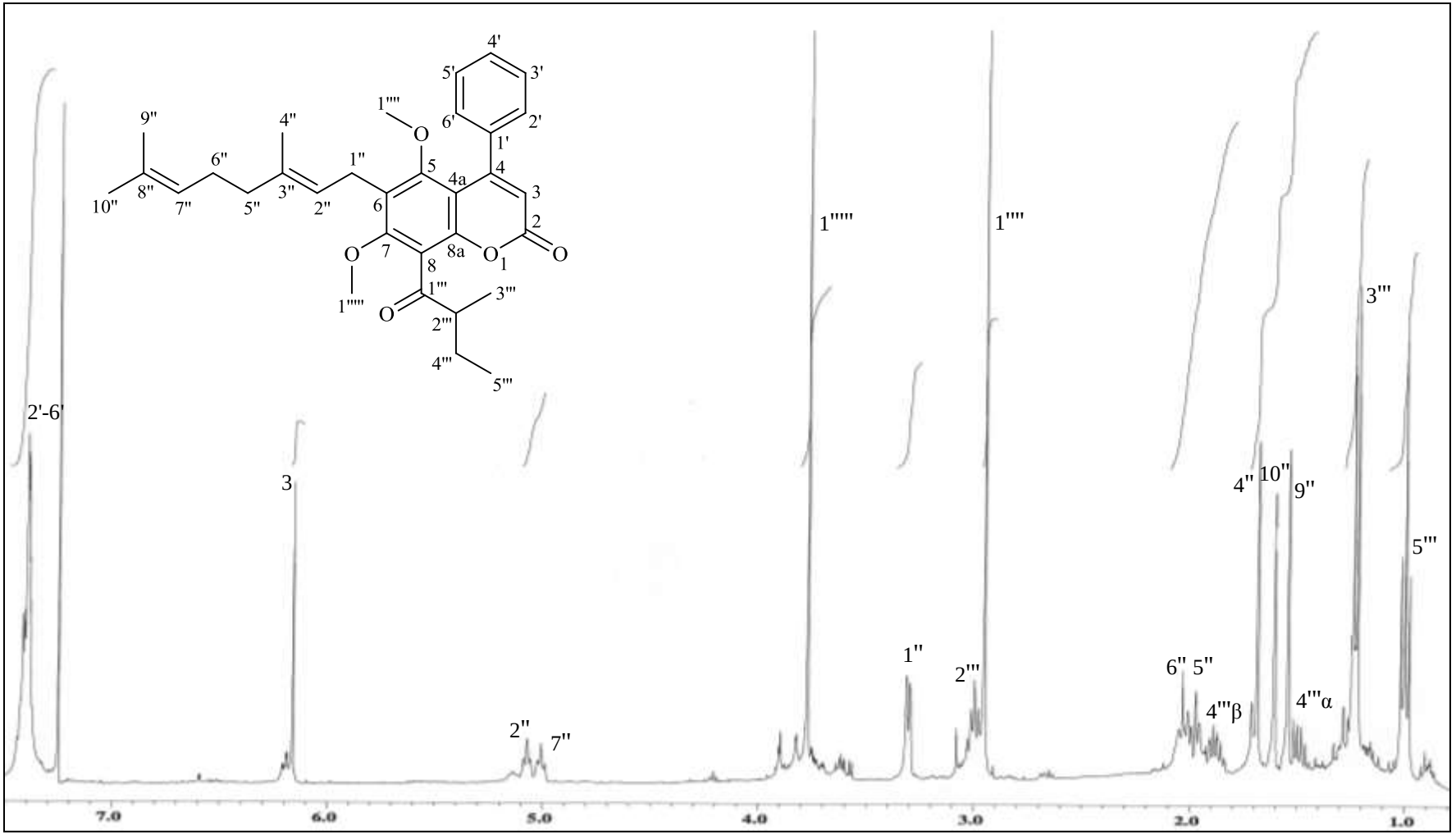


Figure 4.20: ^{1}H NMR Spectrum of Compound M3 **217**

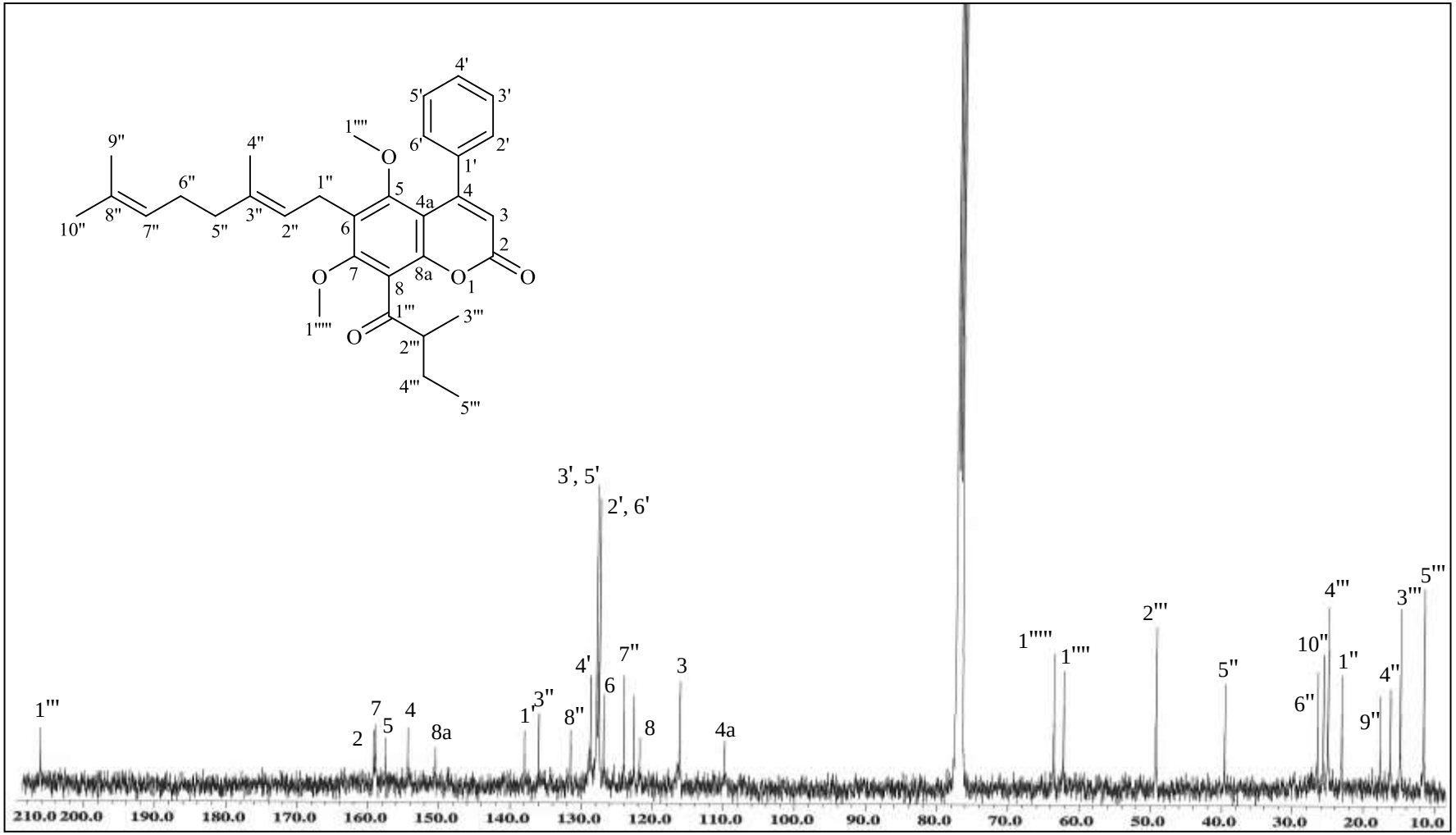
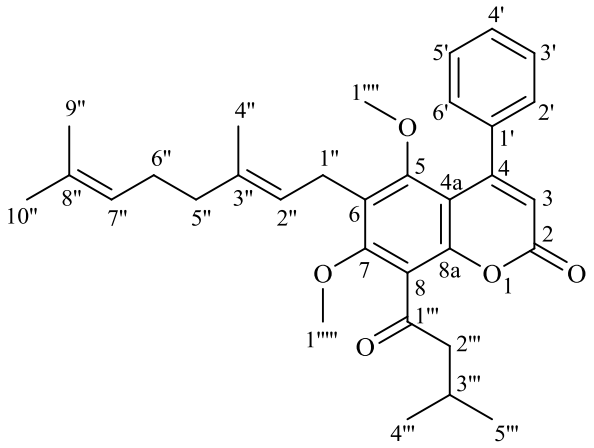


Figure 4.21: ^{13}C NMR Spectrum of Compound M3 **217**

4.2.13 Compound M4: 5,7-Dimethoxy-6-[(*E*)-(3,7-dimethylocta-2,6-dienyl]-8-(3-methylbutanoyl)-4-phenyl-2*H*-chromen-2-one 218



218

Compound M4 was isolated by using HPLC as a white amorphous powder after being methylated from the parent compound J. The molecular formula of $C_{32}H_{38}O_5$ was obtained from the HRESIMS spectrum of compound M4, which exhibited a pseudomolecular $[M+Na]^+$ ion at *m/z* 525.2603 (calculated 525.2617). The UV absorbance of compound M4 was similar to that the parent compound J, with intense absorption bands at 223, 297, 333 nm. The IR spectrum showed absorptions at υ_{max} 1730 cm^{-1}, indicating the presence of an α,β-unsaturated lactone group. However, the IR spectrum of compound M4 did not show the presence of the absorptions of hydroxyl group, which signifies that the hydroxyl groups in the parent compound J had been methylated.

The ^{13}C NMR spectrum of compound M4 displayed thirty-two carbon signals (seven methyls, four methylenes, nine methines and twelve quaternary carbons), as compared to the parent compound J which had thirty carbons signals. Two methoxy singlets were apparent in the ^1H NMR spectrum of compound M4 at δ 2.95 (3H, *s*, H_3-1'''') and δ 3.79

(3H, *s*, H_3-1""), in addition to the missing signals of the 5-OH and 7-OH as compared to the parent compound J.

The observations from the spectral data of compound M4 led to the identification of this compound as 5,7-dimethoxy-6-[(*E*)-(3,7-dimethylocta-2,6-dienyl]-8-(3-methylbutanoyl)-4-phenyl-2*H*-chromen-2-one **218**.

Table 4.16: ^{1}H NMR, ^{13}C NMR, COSY and HMBC (in $CDCl_3$, 400 MHz) of Compound M4 **218**

Position	δ_H, *J* (Hz)	δ_C	COSY	HMBC
2	-	159.3		
3	6.16 (1H, *s*)	116.2		2, 4a, 1'
4	-	154.4		
4a	-	109.8		
5	-	157.5		
6	-	126.9		
7	-	158.8		
8	-	122.2		
8a	-	150.4		
1'	-	138.0		
2'	7.37 (1H, *m*, Ar)	127.6		1', 3'-6'
3'	7.41 (3H, *m*, Ar)	128.0		1', 2', 6'
4'		128.8		
5'		128.0		
6'	7.37 (1H, *m*, Ar)	127.6		1', 3'-6'
1"	3.29 (2H, *d*, *J* = 6.4)	23.1	4"	6, 7, 2", 3"
2"	5.07 (1H, br*t*, *J* = 6.4)	122.7	1"	1", 4", 5"
3"	-	136.1		
4"	1.69 (3H, *s*)	16.3		2", 3", 5"
5"	1.95 (2H, *m*)	39.6		2", 3", 4", 6"
6"	2.01 (2H, *m*)	26.6		3", 5", 7", 8"
7"	5.01 (1H, br*t*, *J* = 6.8)	124.1	6", 9", 10"	9", 10"
8"	-	131.6		
9"	1.55 (3H, *s*)	17.7		7'', 8'', 10''
10"	1.61 (3H, *s*)	25.8		7'', 8'', 9''
1'''	-	202.3		
2'''	2.82 (2H, *d*, *J* = 6.8)	54.2	3'''	1''', 3''', 4''', 5'''
3'''	2.34 (1H, *m*)	24.2	4''', 5'''	1''', 2''', 4''', 5'''
4'''	1.04 (6H, *d*, *J* = 6.4)	22.7		2''', 3''', 5'''
5'''		22.7		2''', 3''', 4'''
1''''	2.95 (3H, *s*)	62.3		
1'''''	3.79 (3H, *s*)	63.5		

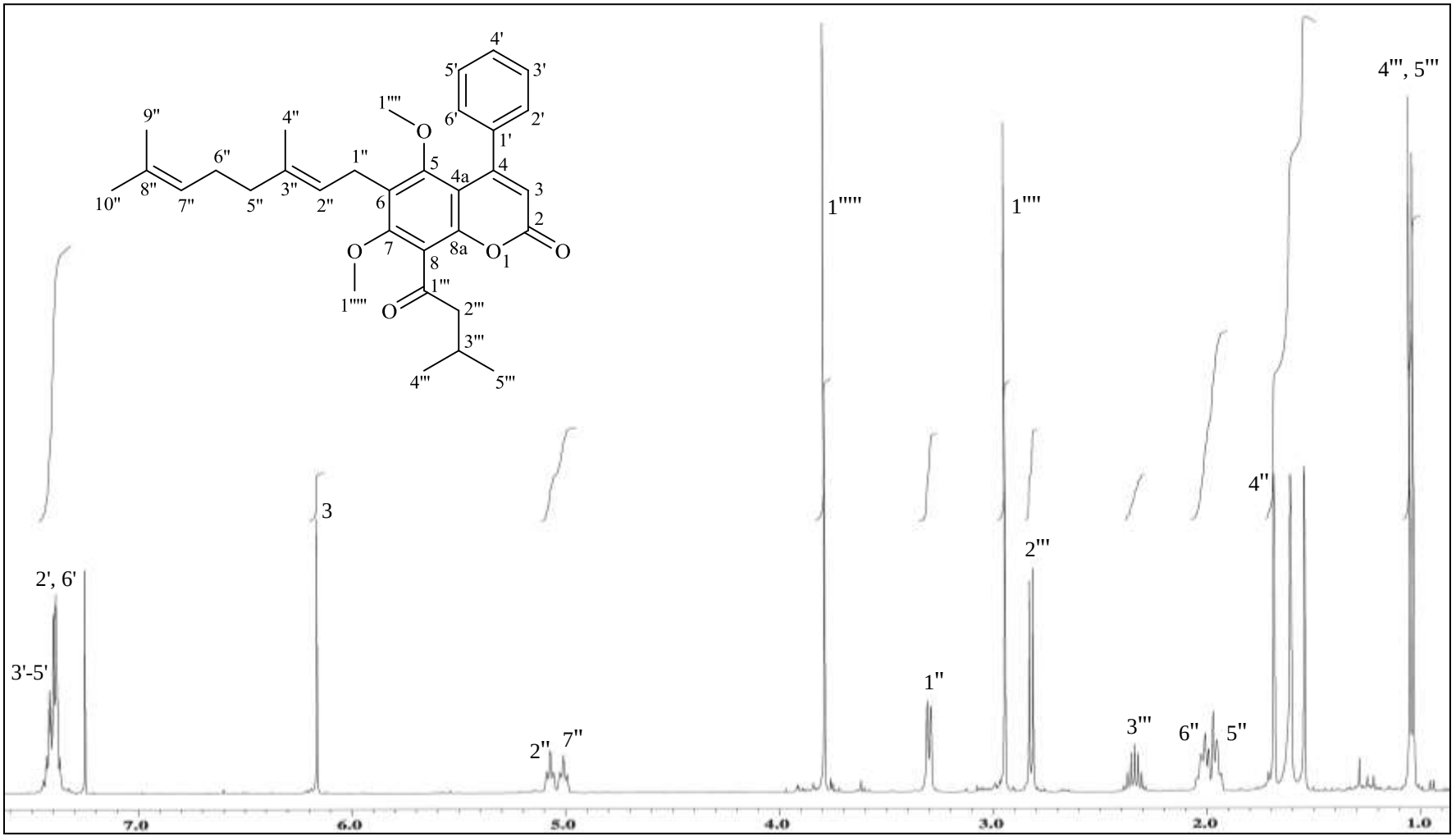


Figure 4.22: ^{1}H NMR Spectrum of Compound M4 **218**

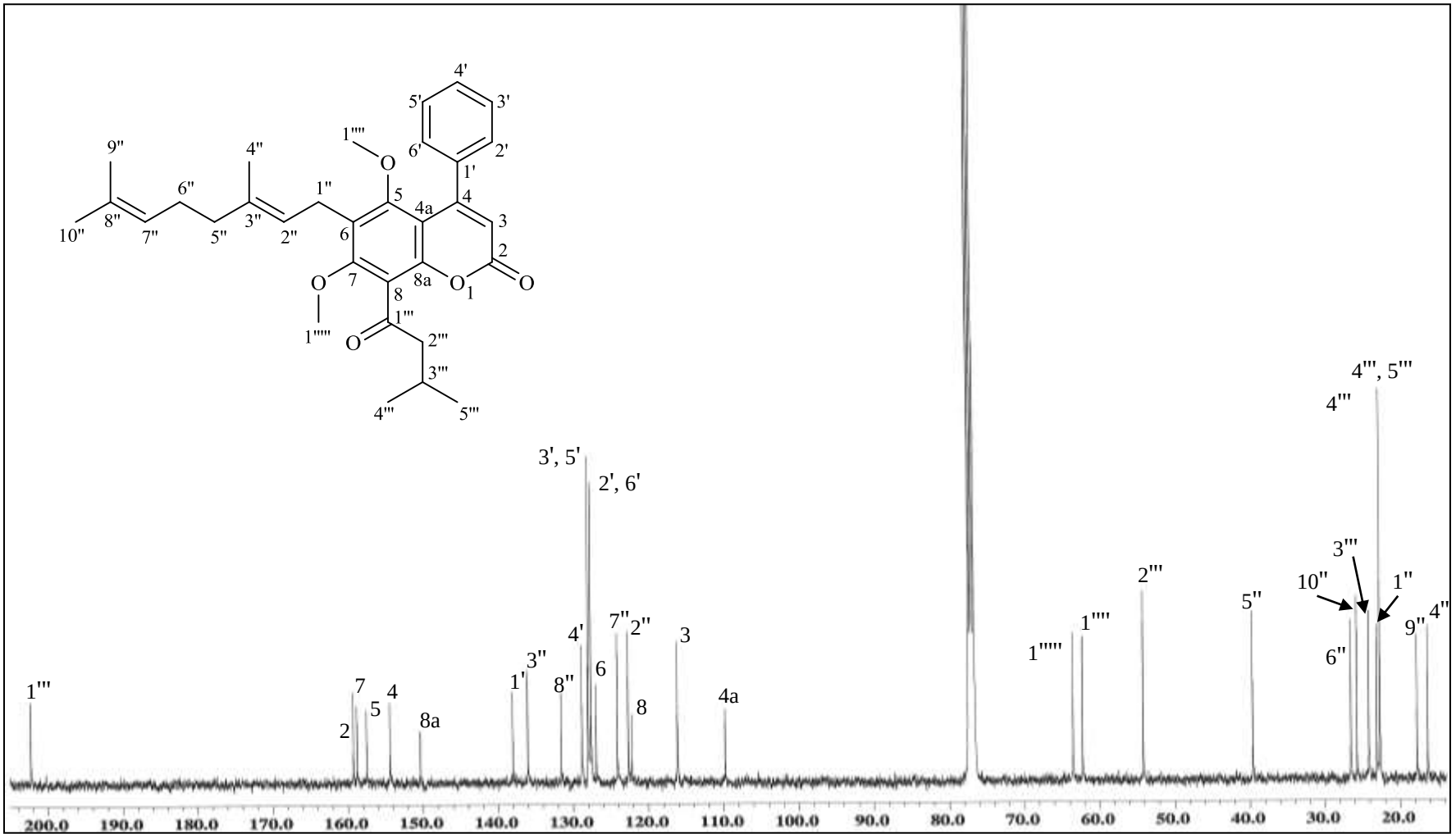


Figure 4.23: ^{13}C NMR Spectrum of Compound M4 **218**

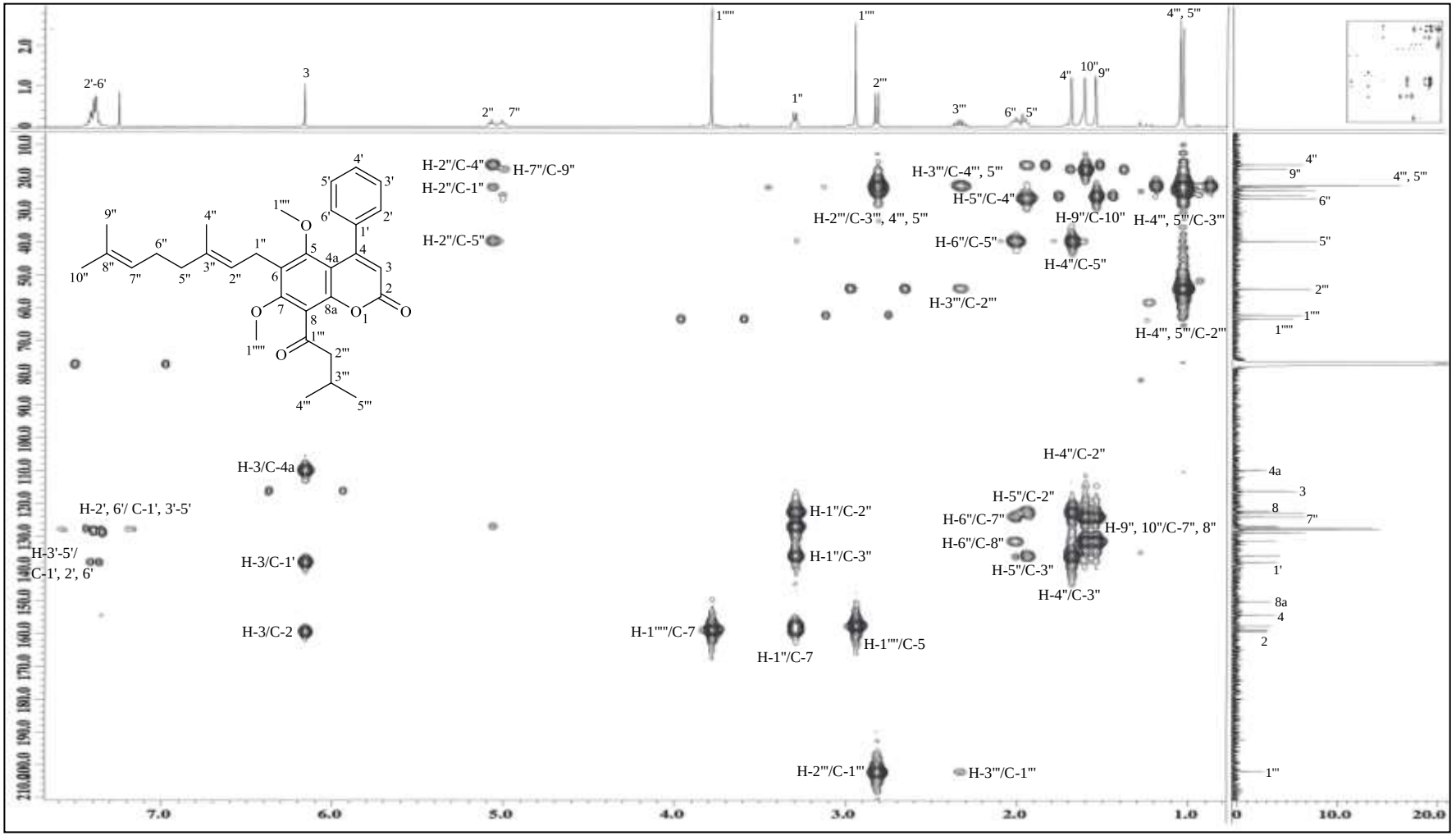
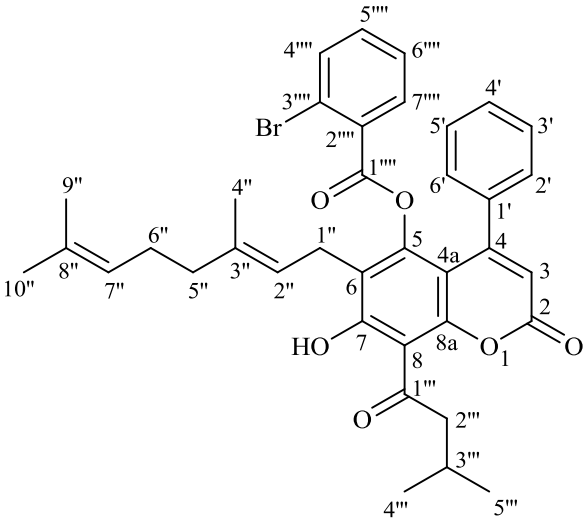


Figure 4.24: HMBC NMR Spectrum of Compound M4 **218**

4.2.14 Compound B1: (*E*)-6-(3,7-Dimethylocta-2,6-dienyl)-7-hydroxy-8-(3-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl 2-bromobenzoate 219



219

Compound B1 was purified by using HPLC as a yellow oil after being bromobenzoylated. The HRESIMS spectrum exhibited a pseudomolecular $[M+Na]^+$ ion at *m/z* 679.1543 (calculated 679.1671), suggesting a molecular formula of $C_{37}H_{37}BrO_6$. The experimental mass seems to be far away from accuracy. Due to the fact that this was a semi-synthetic derivative, low accuracy was still accepted and other data were continued to be analysed, which supported the structural features of compound B1. The IR spectrum of compound B1 exhibited a strong absorption at υ_{max} 1731 cm^{-1}, indicating the presence of α-pyrone and ester groups. An IR absorption peak at υ_{max} 3483 cm^{-1} was also apparent in the IR spectrum of compound B1, which suggested the presence of a hydroxyl group. The UV spectrum (EtOH) of compound B1 displayed absorptions at 221, 300 and 330 nm, which showed a resemblance to the UV absorbance of the parent compound J (227, 299 and 332 nm), supporting the same skeletal structure.

Thirty-seven carbon signals were apparent in the ^{13}C NMR spectrum of compound B1 (five methyls, four methylenes, thirteen methines and fifteen quaternary carbons),

whereas the ^{13}C NMR spectrum of the parent compound J showed thirty carbon signals. This indicated an additional seven carbon signals in compound B1, signifying the addition of the benzoyl group in the structure.

In the ^{1}H NMR spectrum of compound B1, the resonance for the 5-OH in the parent compound J, was absent. However, the resonance for the 7-OH was observed at δ 14.45 in the ^{1}H NMR spectrum of compound B4. With 2-bromobenzoyl chloride as the starting material for this benzoylation, it was ascertained that the bromobenzoylation took place at position 5-OH, and not at 7-OH. In the ^{1}H NMR spectrum of compound B1, evidence for the presence of a 2-bromobenzoyl moiety was also found at δ 7.48 (1H, *d*, *J* = 8.3 Hz, H-4''''), 7.17 (1H, *t*, *J* = 8.3 Hz, H-5''''), 7.30 (1H, *t*, *J* = 8.3 Hz, H-6'''') and 7.57 (1H, *d*, *J* = 8.3 Hz, H-7'''').

Inspection of the spectral data established the identity of compound B1 as (*E*)-6-(3,7-dimethylocta-2,6-dienyl)-7-hydroxy-8-(3-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl 2-bromobenzoate **219**.

Table 4.17: ^{1}H NMR and ^{13}C NMR (in $CDCl_3$, 400 MHz) of Compound B1 **219**

Position	δ_H, *J* (Hz)	δ_C
2	-	161.3
3	6.05 (1H, *s*)	114.1
4	-	158.4
4a	-	105.7
5	-	150.4
6	-	114.2
7-OH	14.45 (1H, *s*)	155.1
8	-	108.2
8a	-	154.5
1'	-	137.9
2'	7.06 (1H, br*d*)	127.6
3'	7.23 (1H, *t*, *J* = 7.8)	128.0
4'	6.77 (1H, *t*, *J* = 7.8)	128.5
5'	7.23 (1H, *t*, *J* = 7.8)	128.0
6'	7.06 (1H, br*d*)	127.6
1"α	3.16 (1H, br*dd*, *J* = 5.9, 14.6)	22.9
1"β	3.40 (1H, br*dd*, *J* = 5.9, 14.6)	
2"	5.00 (1H, br*s*)	120.3
3"	-	136.7
4"	1.62 (3H, *s*)	16.2
5"	1.85 (2H, *m*)	39.5
6"	1.92 (2H, *m*)	26.5
7"	5.00 (1H, br*s*)	124.1
8"	-	131.4
9"	1.49 (3H, *s*)	17.7
10"	1.53 (3H, *s*)	25.4
1'"	-	206.9
2'"	3.28 (2H, *dd*, *J* = 7.3, 7.3)	53.9
3'"	2.38 (1H, *m*)	25.6
4'"	1.08 (6H, *d*, *J* = 6.8)	22.7
5'"		22.7
1""	-	166.3
2""	-	133.9
3""	-	121.6
4""	7.48 (1H, *d*, *J* = 8.3)	133.8
5""	7.17 (1H, *t*, *J* = 8.3)	133.0
6""	7.30 (1H, *t*, *J* = 8.3)	135.1
7""	7.57 (1H, *d*, *J* = 8.3)	126.7

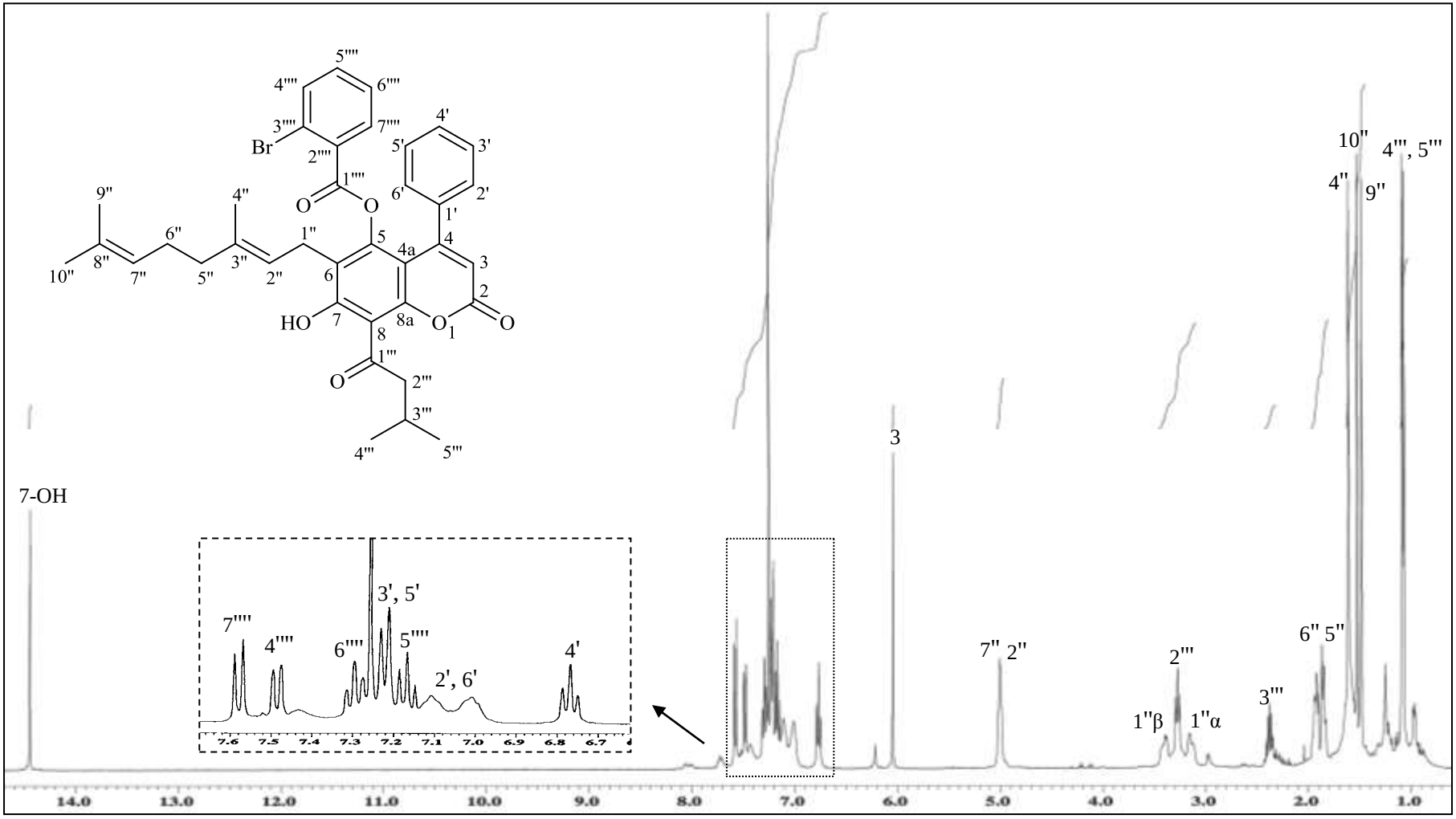


Figure 4.25: ^{1}H NMR Spectrum of Compound B1 **219**

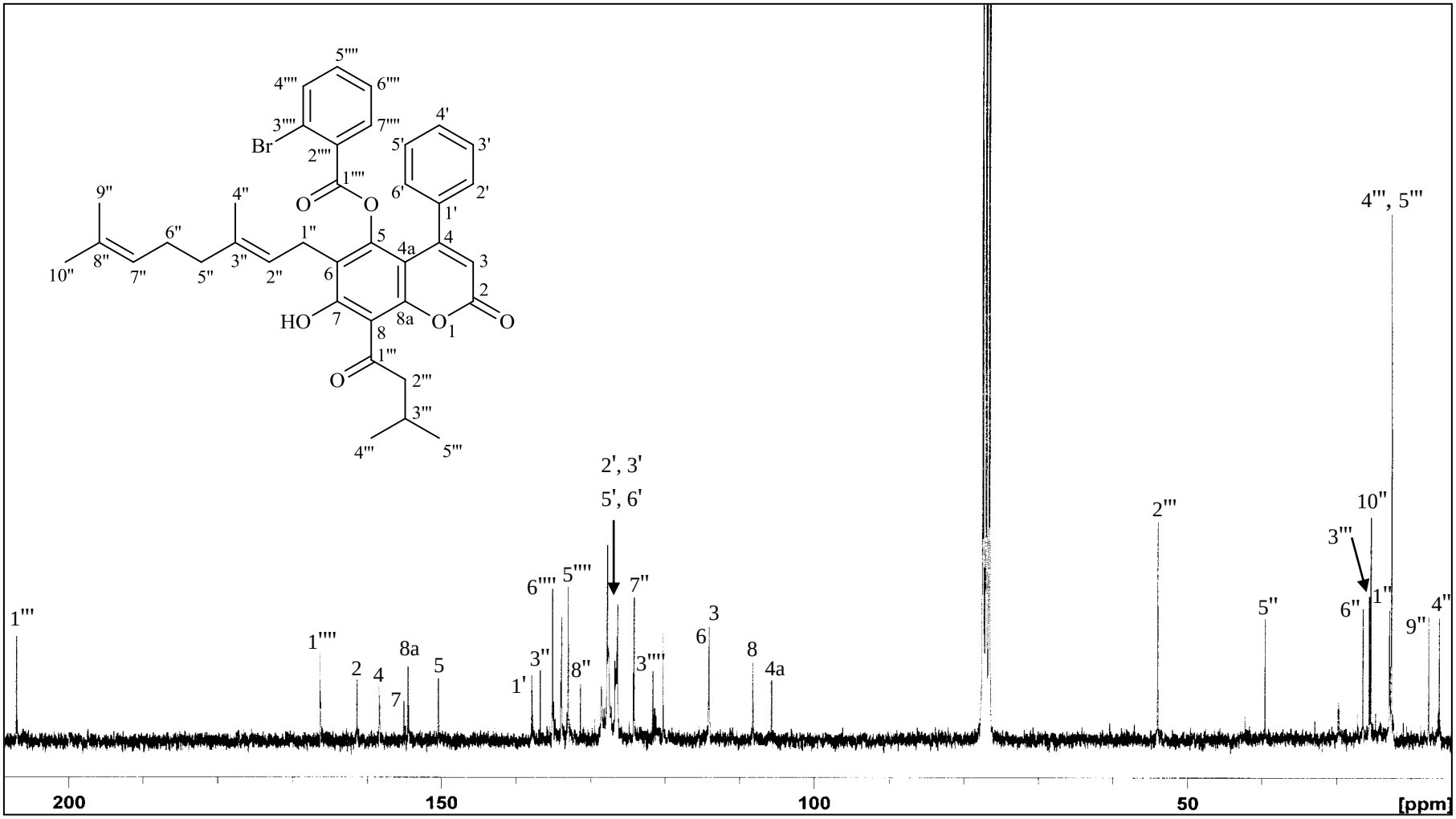


Figure 4.26: ^{13}C NMR Spectrum of Compound B1 **219**

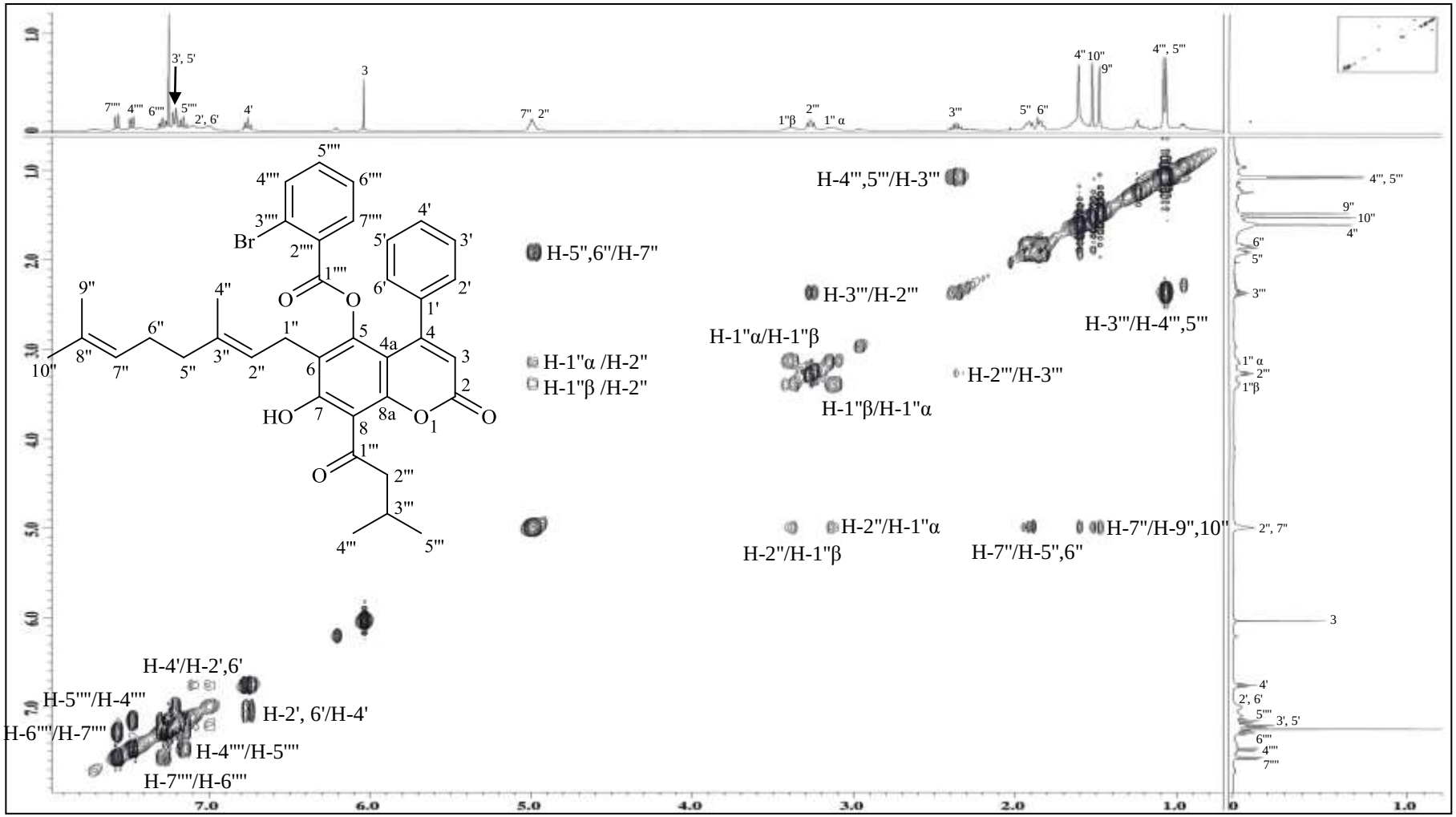
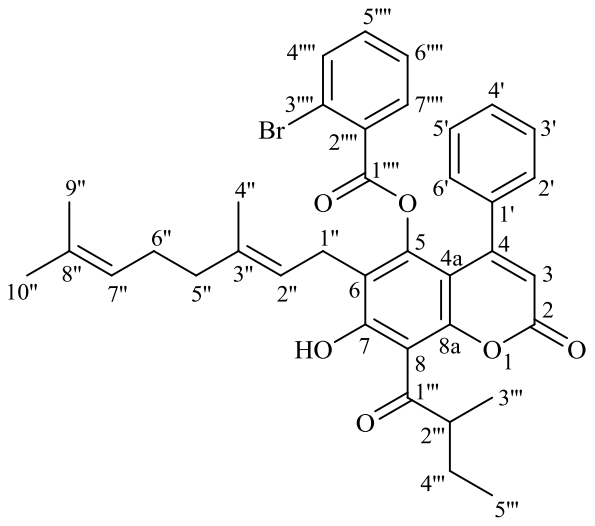


Figure 4.27: COSY Spectrum of Compound B1 **219**

4.2.15 Compound B2: (*E*)-6-(3,7-Dimethylocta-2,6-dienyl)-7-hydroxy-8-(2-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl 2-bromobenzoate 220



220

Compound I which was part of a misture of fraction F1-2, was subjected for bromobenzoylation, and compound B2 was the product of this semi-synthetic process. Compound B2 was isolated as a yellow oil with the molecular formula $C_{37}H_{37}BrO_6$, which was obtained from the HRESIMS measurement which showed a pseudomolecular $[M+Na]^+$ ion at *m/z* 679.1439 (calculated 679.1671). The experimental mass seems to be far away from accuracy. Due to the fact that this was a semi-synthetic derivative, low accuracy was still accepted and other data were continued to be analysed, which supported the structural features of compound B2. The UV spectrum (EtOH) of compound B2 was reminiscent to the parent compound I, with absorption peaks at 225, 298 and 333 nm. The IR spectrum of compound B2 displayed absorption peaks at υ_{max} 1731 (α-pyrone and ester groups) and 3483 cm^{-1} (hydroxyl group), which were similar to the parent compound I.

In the ^1H NMR spectrum of compound B2, the singlet for 7-OH was observed at δ 14.29, whereas the singlet for 5-OH was absent. However, additional signals were

apparent in the aromatic region of the ^{1}H NMR spectrum of compound B2 at δ 7.41 (1H, *dd*, *J* = 1.7, 7.9 Hz, H-4""), 7.23 (1H, *dt*, *J* = 1.7, 7.8 Hz, H-5""), 7.19 (1H, *dt*, *J* = 1.7, 7.8 Hz, H-6"") and 7.50 (1H, *dd*, *J* = 1.7, 7.9 Hz, H-7""). These observations suggested that benzoylation has occurred at the 5-OH, instead of at the 7-OH. The proton of 7-OH is strongly hydrogen bonded with the nearby ketone group (C-1'"), making it more difficult to be benzoylated.

These observations led to the establishment of the structure of compound B2 as (*E*)-6-(3,7-dimethylocta-2,6-dienyl)-7-hydroxy-8-(2-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromen-5-yl 2-bromobenzoate **220**.

Table 4.18: ^{1}H NMR (in $CDCl_3$, 400 MHz) of Compound B2 **220**

Position	δ_H, *J* (Hz)
2	-
3	5.99 (1H, *s*)
4	-
4a	-
5	-
6	-
7-OH	14.29 (1H, *s*)
8	-
8a	-
1'	-
2'	6.96 (1H, br*d*)
3'	7.10 (1H, *dt*, *J* = 1.2, 7.7)
4'	6.70 (1H, *dt*, *J* = 1.2, 7.5)
5'	7.10 (1H, *dt*, *J* = 1.2, 7.7)
6'	6.96 (1H, br*d*)
1"α	3.10 (1H, br*s*)
1"β	3.31 (1H, br*s*)
2"	4.94 (1H, br*s*)
3"	-
4"	1.55 (3H, *s*)
5"	1.76 (2H, *m*)
6"	1.81 (2H, *m*)
7"	4.94 (1H, br*s*)
8"	-
9"	1.42 (3H, *s*)
10"	1.47 (3H, *s*)
1'"	-
2'"	3.93 (1H, *m*)
3'"	1.25 (2H, *d*, *J* = 6.7)
4'"α	1.40 (1H, *m*)
4'"β	1.74 (1H, *m*)
5'"	0.98 (3H, *t*, *J* = 7.4)
1""	-
2""	-
3""	-
4""	7.41 (1H, *dd*, *J* = 1.7, 7.9)
5""	7.23 (1H, *dt*, *J* = 1.7, 7.8)
6""	7.19 (1H, *dt*, *J* = 1.7, 7.8)
7""	7.50 (1H, *dd*, *J* = 1.7, 7.9)

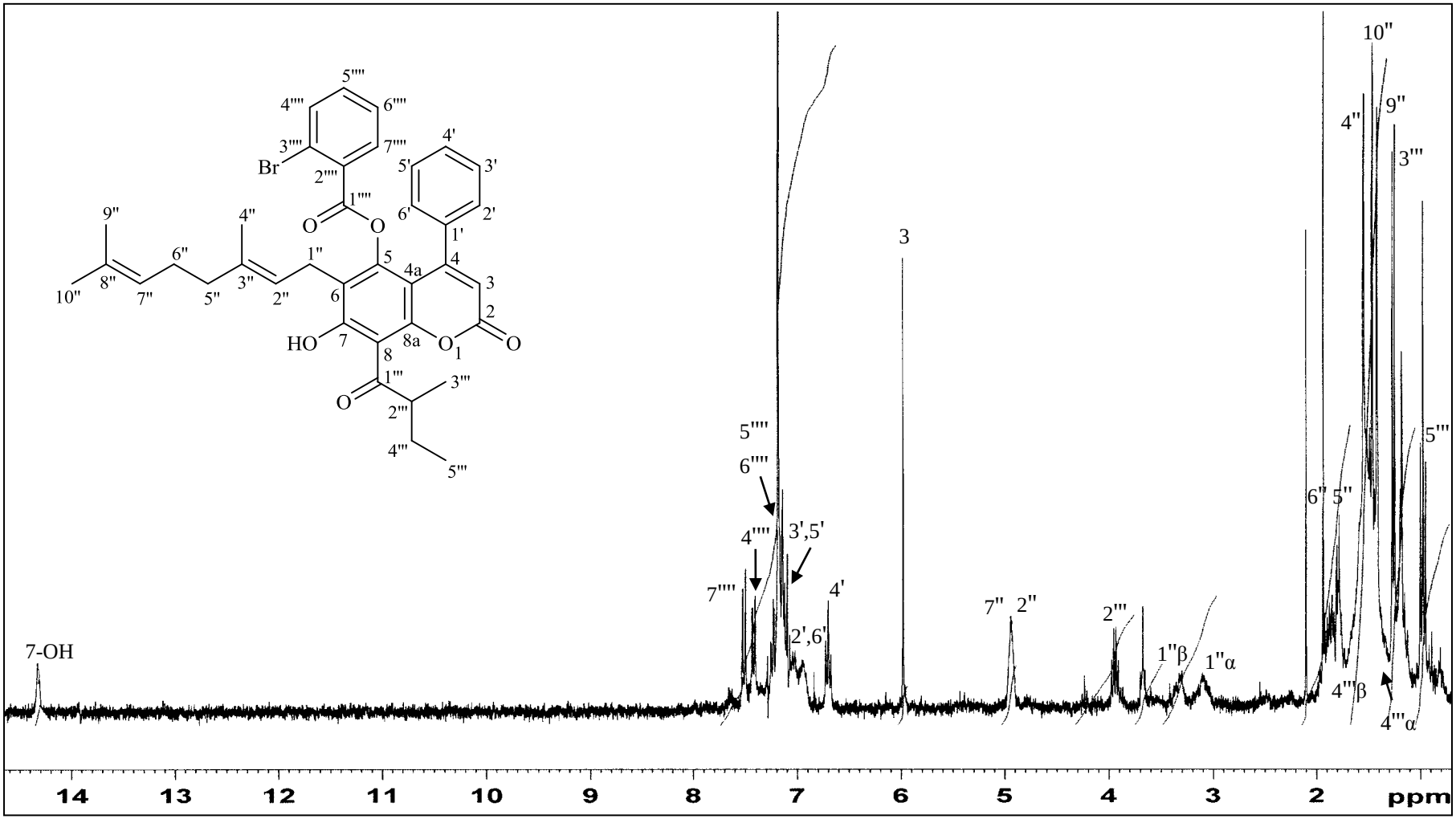
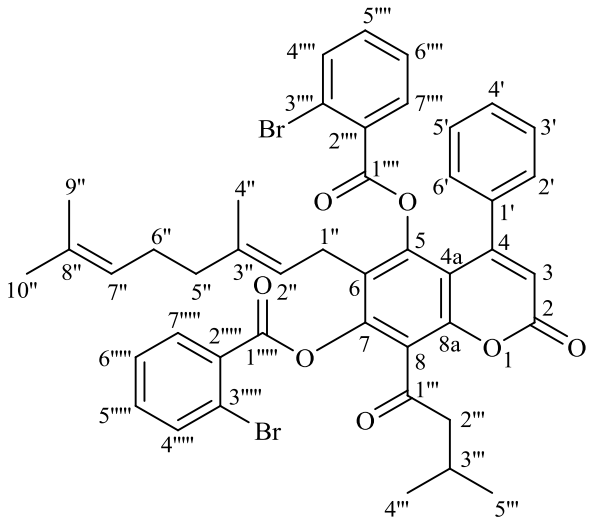


Figure 4.28: ^{1}H NMR Spectrum of Compound B2 **220**

4.2.16 Compound B3: (*E*)-6-(3,7-Dimethylocta-2,6-dienyl)-8-(3-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl bis(2-bromobenzoate) 221



221

Compound B3 was bromobenzoylated from the parent compound J which was in a mixture, and was purified by using HPLC as a yellowish oil. The molecular formula of $C_{44}H_{40}Br_2O_7$ of compound B3 was established from the HRESIMS spectrum which showed a pseudomolecular $[M+Na]^+$ ion at *m/z* 861.0873 (calculated 861.1038). The IR spectrum of compound B3 displayed an absorption peak at υ_{max} 1731 cm^{-1}, indicating the presence of α-pyrone and ester groups. On the contrary, an absorption peak indicating the presence of hydroxyl group was absent in the IR spectrum of compound B3, suggesting the bromobenzoylation of both hydroxyl groups. The UV absorbance peaks (EtOH) at 225, 297 and 330 nm of compound B3 resembled the UV spectrum of the parent compound J, which supported the same skeletal structure.

The signals of the 5-OH and 7-OH were absent in the ^1H NMR spectrum of compound B3, as compared to the parent compound J. This observation supported the absence of hydroxyl group peaks in the IR spectrum of compound B3. Moreover, the aromatic region showed more complicated proton signals, suggesting the presence of two 2-

bromobenzene unit in compound B3; a set of δ 7.51 (1H, *d*, *J* = 8.3 Hz, H-4""), 7.23 (1H, *t*, *J* = 8.3 Hz, H-5""), 7.30 (1H, *t*, *J* = 8.3 Hz, H-6"") and 7.57 (1H, *d*, *J* = 8.3 Hz, H-7""), of the 2-bromobenzene unit attached to C-5, which appears to be more upfield than the 2-bromobenzene unit attached to C-7; δ 7.73 (1H, *dd*, *J* = 2.4, 7.0 Hz, H-4""'), 7.45 (1H, *t*, *J* = 8.0 Hz, H-5""'), 7.42 (1H, *t*, *J* = 8.0 Hz, H-6""') and 8.07 (1H, *dd*, *J* = 2.4, 7.0 Hz, H-7""').

The observed spectral data of compound B3, and comparison with the parent compound J, led to the identification of this compound as (*E*)-6-(3,7-dimethylocta-2,6-dienyl)-8-(3-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl bis(2-bromobenzoate) **221**.

Table 4.19: ^{1}H NMR and ^{13}C NMR (in $CDCl_3$, 400 MHz) of Compound B3 **221**

Position	δ_H, *J* (Hz)	δ_C
2	-	158.1
3	6.22 (1H, *s*)	112.3
4	-	153.3
4a	-	118.1
5	-	147.2
6	-	124.2
7	-	150.3
8	-	118.1
8a	-	148.6
1'	-	136.9
2'	7.12 (1H, br*d*)	127.6
3'	7.18 (1H, *t*, *J* = 7.8)	128.6
4'	6.79 (1H, *t*, *J* = 7.8)	129.4
5'	7.18 (1H, *t*, *J* = 7.8)	128.6
6'	7.12 (1H, br*d*)	127.6
1"α	3.13 (1H, br*d*, *J* = 13.5)	} 24.3
1"β	3.36 (1H, br*d*, *J* = 13.5)	
2"	4.95 (1H, brs)	120.0
3"	-	137.0
4"	1.60 (3H, *s*)	16.3
5"	1.75 (2H, *m*)	39.3
6"	1.83 (2H, *m*)	26.3
7"	4.95 (1H, brs)	123.9
8"	-	131.4
9"	1.23 (3H, *s*)	17.6
10"	1.51 (3H, *s*)	25.6
1'''	-	200.0
2'''	2.98 (2H, *d*, *J* = 6.7)	53.2
3'''	2.30 (1H, *m*)	24.2
4'''	} 0.98 (6H, *d*, *J* = 6.7)	22.5
5'''		22.5
1''''	-	161.5
2''''	-	132.6
3''''	-	122.9
4''''	7.51 (1H, *d*, *J* = 8.3)	133.8
5''''	7.23 (1H, *t*, *J* = 8.3)	135.1
6''''	7.30 (1H, *t*, *J* = 8.3)	127.4
7''''	7.57 (1H, *d*, *J* = 8.3)	126.3
1'''''	-	162.9
2'''''	-	133.1
3'''''	-	123.0
4'''''	7.73 (1H, *dd*, *J* = 2.4, 7.0)	133.9
5'''''	7.45 (1H, *t*, *J* = 8.0)	135.3
6'''''	7.42 (1H, *t*, *J* = 8.0)	127.6
7'''''	8.07 (1H, *dd*, *J* = 2.4, 7.0)	126.4

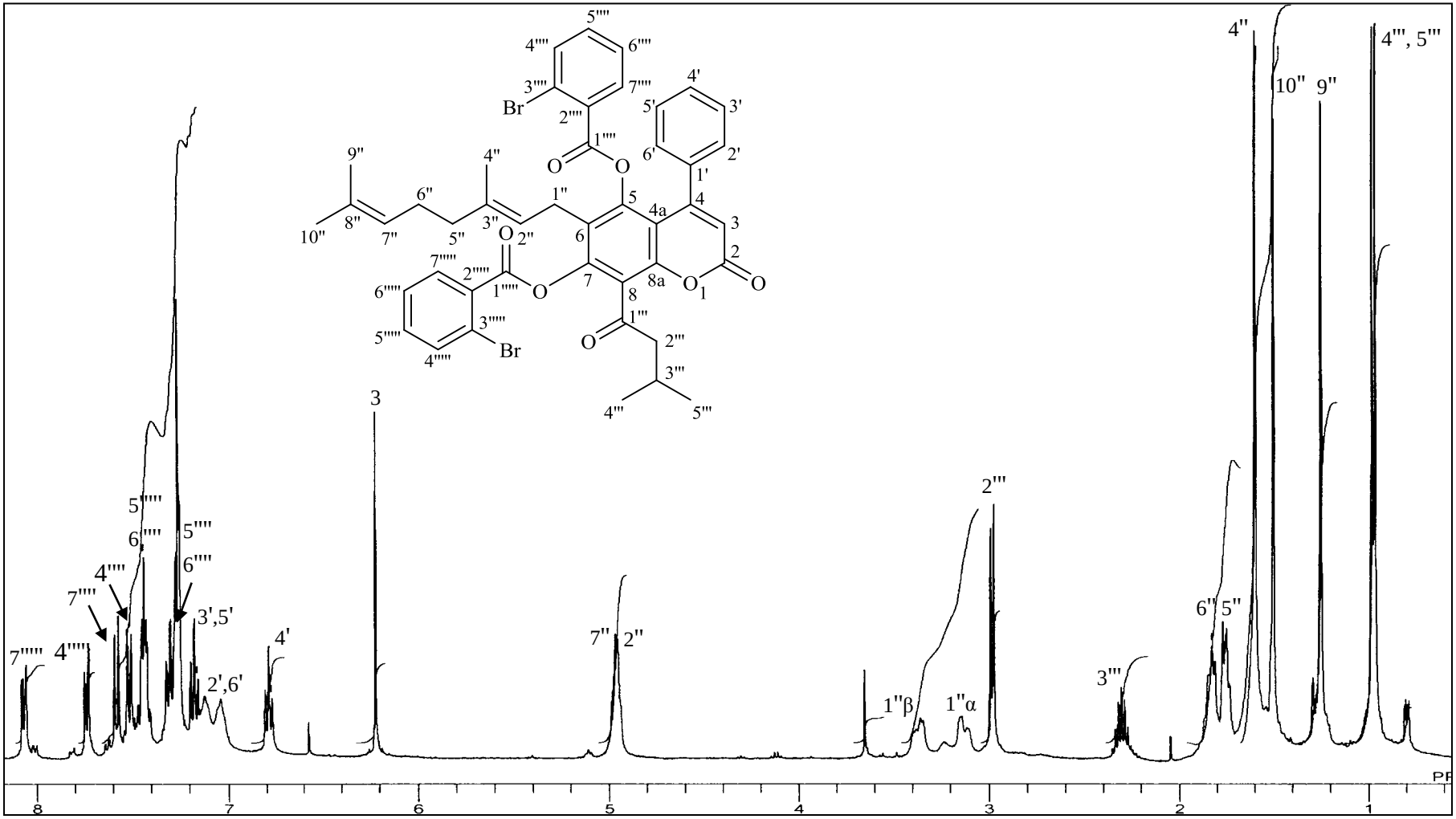


Figure 4.29: ^{1}H NMR Spectrum of Compound B3 **221**

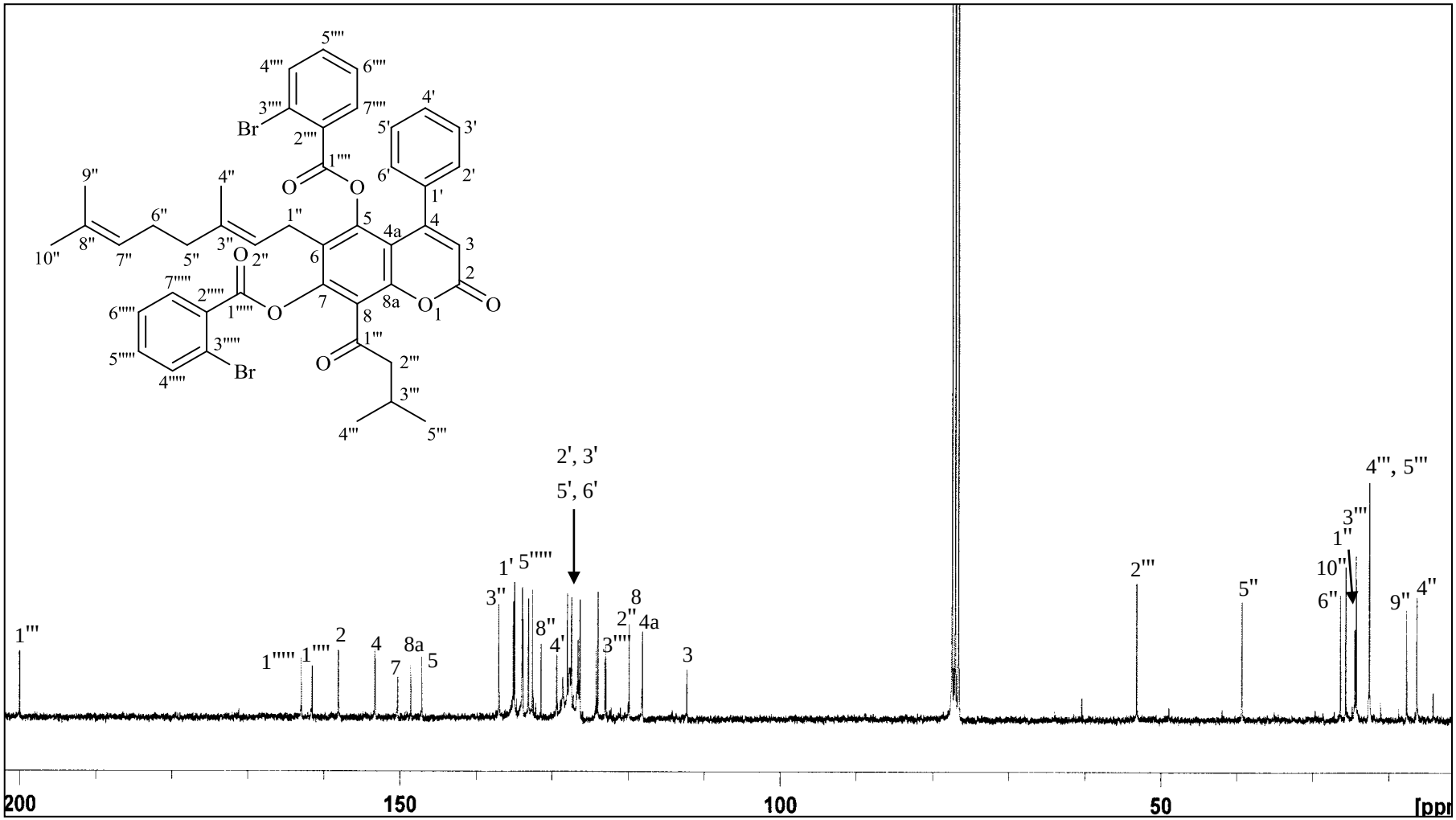
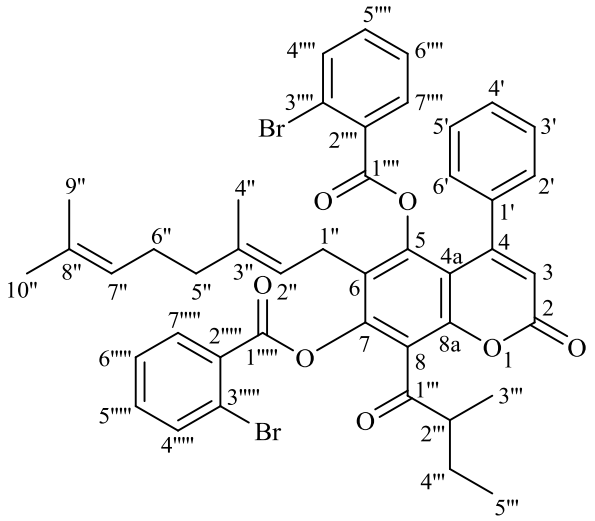


Figure 4.30: ^{13}C NMR Spectrum of Compound B3 **221**

4.2.17 Compound B4: (*E*)-6-(3,7-Dimethylocta-2,6-dienyl)-8-(2-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl bis(2-bromobenzoate) 222



222

Compound B4 was isolated through HPLC as a yellowish oil after being bromobenzoylated from the parent compound I which was in a mixture of fraction F1-2. The HRESIMS spectrum of compound B4 revealed a pseudomolecular $[M+Na]^+$ ion at *m/z* 861.0733 (calculated 861.1038), signifying the molecular formula $C_{44}H_{40}Br_2O_7$. The same skeletal structure of compound B4 with the parent compound I was supported by the UV absorbance peaks (EtOH) of compound B4 at 225, 297 and 330 nm. This observation was further supported by the IR at υ_{max} 1731 cm^{-1}, which indicated the presence of α-pyrone and ester groups.

The complex signals of the 2-bromobenzene protons in the aromatic region of the ^1H NMR spectrum of compound B4 indicated that both hydroxyl groups were bromobenzoylated, supported by the absence of the 5-OH and 7-OH resonances, as compared to the parent compound I. These spectral data observations led to the identification of compound B4 as (*E*)-6-(3,7-dimethylocta-2,6-dienyl)-8-(2-methylbutanoyl)-2-oxo-4-phenyl-2*H*-chromene-5,7-diyl bis(2-bromobenzoate) **222**.

Table 4.20: ^{1}H NMR (in $CDCl_3$, 400 MHz) of Compound B4 **222**

Position	δ_H, *J* (Hz)
2	-
3	6.15 (1H, *s*)
4	-
4a	-
5	-
6	-
7	-
8	-
8a	-
1'	-
2'	7.02 (1H, br*d*)
3'	7.11 (1H, *dt*, *J* = 1.2, 7.7)
4'	6.71 (1H, *dt*, *J* = 1.2, 7.5)
5'	7.11 (1H, *dt*, *J* = 1.2, 7.7)
6'	7.02 (1H, br*d*)
1"α	3.08 (1H, br*d*, *J* = 13.5)
1"β	3.30 (1H, br*d*, *J* = 13.5)
2"	4.86 (1H, br*t*, *J* = 8.3)
3"	-
4"	1.53 (3H, *s*)
5"	1.67 (2H, *m*)
6"	1.76 (2H, *m*)
7"	4.91 (1H, br*t*, *J = 8.3*)
8"	-
9"	1.19 (3H, *s*)
10"	1.43 (3H, *s*)
1'''	-
2'''	3.16 (1H, *m*)
3'''	1.16 (2H, *d*, *J* = 5.4)
4'''α	1.37 (1H, *m*)
4'''β	1.64 (1H, *m*)
5'''	0.92 (3H, *t*, *J* = 7.8)
1''''	-
2''''	-
3''''	-
4''''	7.45 (1H, *dd*, *J* = 1.6, 8.0)
5''''	7.25 (1H, *dt*, *J* = 1.8, 7.8)
6''''	7.20 (1H, *dt*, *J* = 1.8, 7.8)
7''''	7.52 (1H, *dd*, *J* = 1.6, 8.0)
1'''''	-
2'''''	-
3'''''	-
4'''''	7.67 (1H, *dd*, *J* = 2.2, 7.0)
5'''''	7.35 (1H, *t*, *J* = 8.0)
6'''''	7.37 (1H, *t*, *J* = 8.0)
7'''''	7.94(1H, *dd*, *J* = 2.2, 7.0)

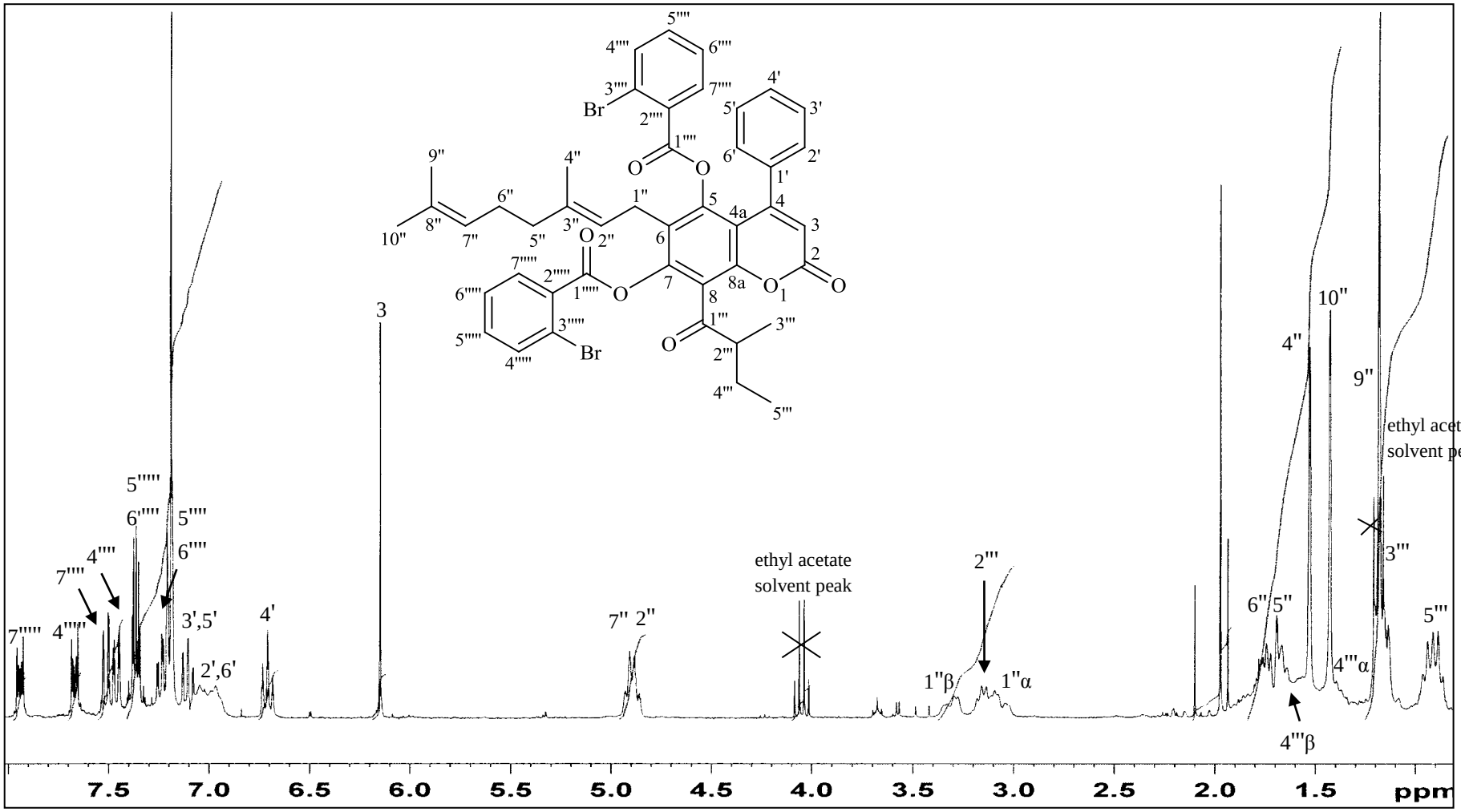


Figure 4.31: ^{1}H NMR Spectrum of Compound B4 **222**

CHAPTER 5

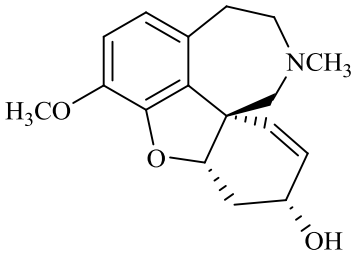
AChE ACTIVITY AND SAR STUDIES OF 4-PHENYLCOUMARINS

Alzheimer's disease (AD), the most common form of dementia in the elderly, is a neurodegenerative disease characterized by progressive memory impairment, cognitive deficits and behavioral changes[96]. In the deaths caused by AD, Finland ranks as number one, with the death rate of 34.9/100000, followed by Iceland and United States, with death rates of 25.1/100,000 and 24.8/100,000, respectively. Malaysia ranks as the 185th country, with a death rate of 0.2/100,000 (data source: WHO, 2011). As general health care improves, and with the increase of the elderly population, the number of AD patients is anticipated to increase dramatically[97].

The molecular causes of this condition remain unknown in spite of the existence of several theories regarding the pathogenesis of AD; cholinergic, amyloid, tau, calcium hypothesis and isoprenoid change[98]. Now, the most widely accepted biochemical theory of the disease is the cholinergic hypothesis, which states that the decline in cognitive and mental functions associated with AD is related to the loss of cortical cholinergic neurotransmission. AD is associated with a loss of the presynaptic markers of the cholinergic system in the brain regions related to memory and learning, and is characterized by the presence of amyloid deposits and neurofibrillary tangles in the brain of afflicted individuals[99]. One coherent way to enhance cholinergic neurotransmission is to inhibit an enzyme responsible for the metabolic breakdown of acetylcholine (ACh). Acetylcholinesterase (AChE) enzyme inhibition is an important target for the management of AD, and AChE inhibitors are the main stay drugs for its management[100].

Within the most successful AChE inhibitors introduced as drugs, small molecules of natural origin have occupied a prominent position. Indeed, two (galanthamine **223** and rivastigmine **224**) out of the three (these two and donepezil **225**) recommended drugs for AD symptomatic treatment have a direct or indirect natural origin[101]. In addition, tacrine **226** and ensaculin **227** also show a modest induction of the improvement in memory and cognitive functions, and have been used to treat AD clinically[102,103]. These have pointed out the significance of exploring nature as a source of bioactive compounds that may serve as the leads or scaffolds for further chemical elaboration.

In line with that, the present work deals with the bioassay-guided isolation of AChE inhibitors from the bark of *M. elegans* (King) Kosterm. The hexane extract from the bark gave 90% inhibition of AChE at 10 μg/mL concentration. The hexane : ethyl acetate (95 : 5) fraction of this active hexane extract gave 100% inhibition at 10 μg/mL concentration. Further isolation and structural elucidation of compounds from this fraction were carried out. In addition to the bioassay-guided isolation of AChE inhibitors, other chemical constituents from *M. elegans* were also isolated, which possessed the same 4-phenylcoumarin skeletal features. In order to obtain more analogues, chemical modifications were performed on the active fraction and the major chemical constituents from this plant. A structure-activity relationship (SAR) study has been carried out with the natural and modified 4-phenylcoumarins. This chapter will discuss the results and the related SAR study of these compounds.



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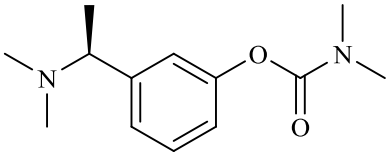

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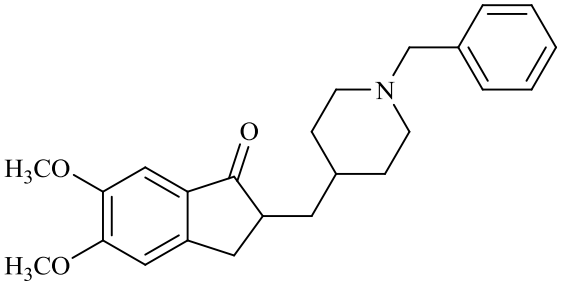

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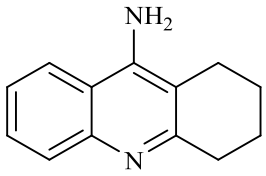

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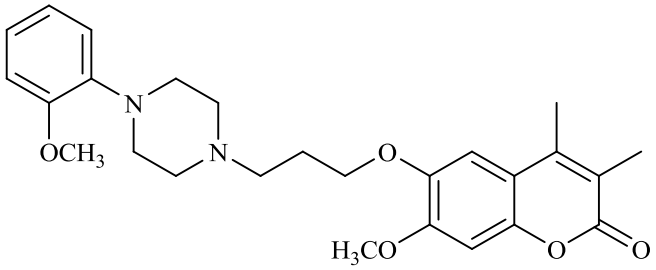

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5.1 Bioassay-guided Study of Acetylcholinesterase Inhibitory Activity of *M. elegans*

The hexane, ethyl acetate and methanol extracts from the bark of *Mesua elegans* were subjected to acetyl cholinesterase (AChE) inhibitory evaluations. The hexane, ethyl acetate and methanol extracts exhibited inhibition percentages of 90%, 80%, and less than 50%, at 10 μg/mL concentration, respectively. The hexane extract was further investigated for its chemical contents since it was the most potent among the extracts.

The hexane extract was fractionated into eight fractions (Table 5.1), in which the fraction hexane : ethyl acetate (95 : 5) (F1) showed the highest inhibition percentage of 100% at 10 μg/mL concentration. Therefore, this active fraction was subjected to further chromatographic separation.

Bioassay-guided isolation of the active compounds from the active fraction resulted in the discovery of nine 4-phenylcoumarins, of which four of them (compounds D, E, I and O) showed significant activity. Compound E **190** gave the highest activity of 80% inhibition at 10 μg/mL concentration and an IC_{50} value of 0.70 μM. For these AChE inhibition evaluations, tacrine was used as the positive control. The inhibition percentage and IC_{50} values of the isolated compounds are listed in Table 5.2. The summary of the bioassay-guided procedure is shown in Scheme 8.1.

Table 5.1: Solvent Ratios Used for the Fractionation of the Crude Hexane Extract and the Inhibition Percentage of Each Fraction

Fraction	Solvent Ratio (Hexane : Ethyl Acetate)	Inhibition Percentage (at 10 μg/mL)
F1	95 : 5	100
F2	90 : 10	Less than 50
F3	80 : 20	
F4	70 : 30	
F5	50 : 50	
F6	0 : 100	
Fraction	Solvent Ratio (Ethyl Acetate : Methanol)	Inhibition Percentage (at 10 μg/mL)
F7	50 : 50	Less than 50
F8	0 : 100	

Table 5.2: Inhibition Percentage and IC_{50} Values of Isolated Compounds

Compounds	Inhibition Percentage (at 10 μg/mL)			IC_{50} (μM)
	Test 1	Test 2	Average	
Compound B	-	-	-	-
Compound D	80	70	75	1.06
Compound E	80	80	80	0.70
Compound G	20	40	30	-
Compound H	20	30	25	-
Compound I	70	70	70	3.06
Compound J	20	40	30	-
Compound M	30	50	40	-
Compound O	70	60	65	8.73
Tacrine	-	-	-	0.074

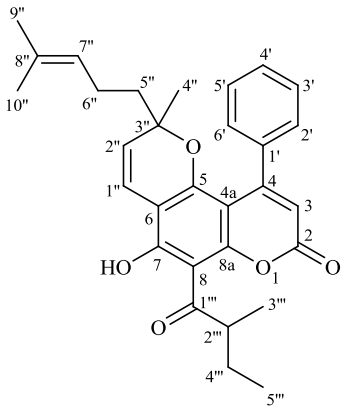
5.2 SAR Studies of 4-Phenylcoumarins as Acethylcholinesterase Inhibitors

A total of twenty-seven 4-phenylcoumarins were evaluated for the inhibition of AChE (at 10 μg/mL), in which ten compounds were natural products and seventeen compounds were semi-synthetic compounds. A SAR (structure-activity relationship) study was carried out with these 4-phenylcoumarins. 4-Phenylcoumarins with above 50% inhibition of AChE were considered for this SAR discussion. From the results obtained (Table 5.3), the author has attempted a SAR study analysis, which will be discussed briefly in the coming paragraphs.

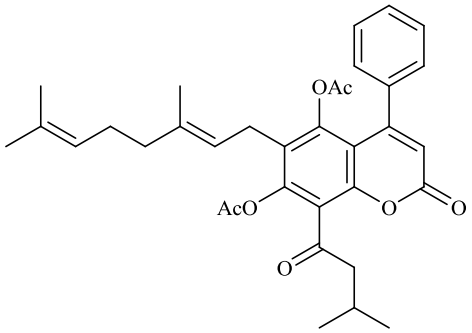
Table 5.3: Average Inhibition Percentage (at 10 ug/mL) of the Natural and Semi-synthesized Compounds

Compound	Average Inhibition %
D	75.0
E	80.0
F	23.0
G	30.0
H	25.0
I	70.0
J	30.0
L	46.0
M	40.0
O	65.0
A1	41.0
A2	15.5
A3	29.5
A4	29.5

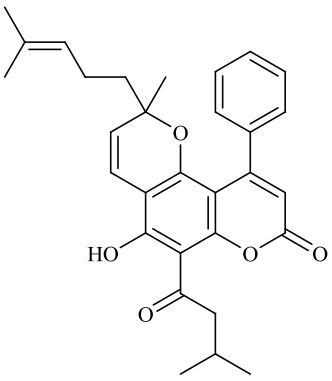
Compound	Average Inhibition %
A5	47.5
A6	58.0
A7	29.5
A8	56.0
A9	76.0
M1	32.0
M2	39.5
M3	24.0
M4	54.5
B1	49.5
B2	39.5
B3	24.5
B4	50.0



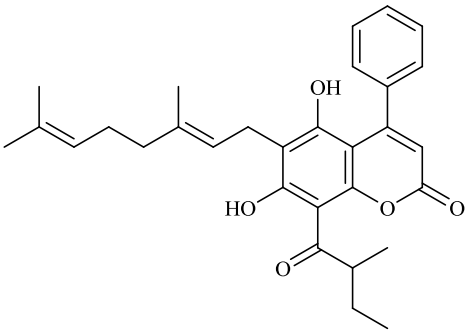
E (80.0%)



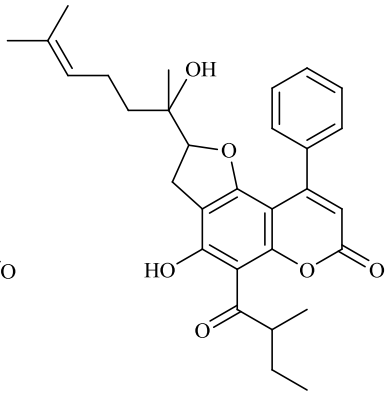
A9 (76.0%)



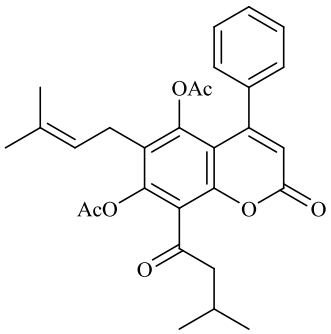
D (75.0%)



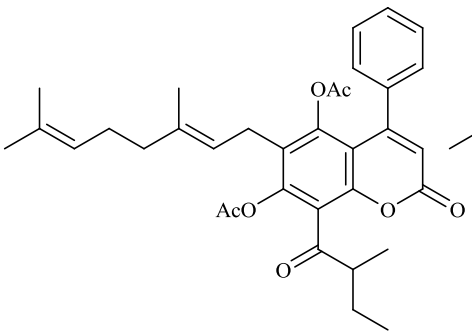
I (70.0%)



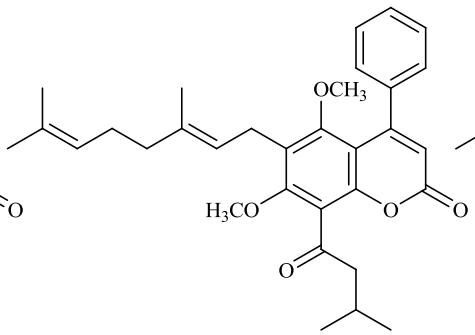
O (65.0%)



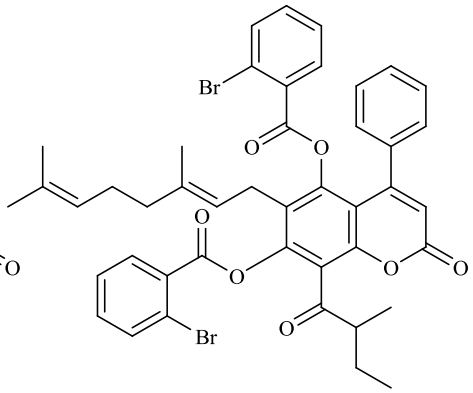
A6 (58.0%)



A8 (56.0%)



M4 (54.5%)



B4 (50.0%)

Figure 5.1: Compounds with AChE Inhibition Above 50% (at 10 μg/mL)

The most active compound was compound E showing 80.0% inhibition at 10 μg/mL, which has a geranyl-pyran cyclized ring at positions C-5/C-6, and a 2-methylbutanoyl at position C-8. The activity dropped a little to 76.0% inhibition in compound A9, which has an open chain geranyl group attached at position C-6 and a 3-methylbutanoyl at position C-8, together with two acetate groups at position C-5 and C-7. Interestingly, the inhibition activity of compound D, which also possesses geranyl pyran substituent (an analogue of compound E), was comparable to compound A9.

Moreover, it is worthy to note that the activity of compound I (70.0%), which has an open chain geranyl at position C-6, has decreased compared to compound E which has a the geranyl-pyran substituent at position C-5/C-6. It was also observed that geranyl-furan substituted compound O was less active than geranyl-pyran substituted compounds E and D. For the furan substituted compounds at position C-5/C-6, it was noted that the prenyl-furan substituted compounds L and M were less active (below 50% of AChE inhibition) than the geranyl-furan substituted compound O (65.0%), indicating that a longer chain is favourable for the activity.

Overall, the natural compounds showed good activity, and one may assume that chemical modification of substituents of 4-phenylcoumarins may induce change in activity. This may be exemplified by the acetylated compound A9 (76.0%) and the methylated compound M4 (54.5%), which were more active than their parent compound J (30.0%). However, chemical modification may also decrease the activity of a more active natural compound. For instance in the case of compound I which has 70.0% AChE inhibition, has shown a decrease in activity upon acetylation (compound A8 - 56.0%) and bromobenzoylation (compound B4 - 50.0%).

Generally, 4-phenylcoumarins with longer or bulkier substituents at position C-6 gave a higher percentage of inhibition as compared to the shorter substituents at the same position. For example, of the nine compounds which had an AChE inhibition activity of more than 50.0%, eight of the 4-phenylcoumarins were substituted by a geranyl or geranyl derivative at position C-5/C-6, as compared to one compound A6, which has a prenyl substituent at position C-6. On the other hand, compounds with a 2-methylbutanoyl or 3-methylbutanoyl substituent at position C-8 were active, whereas compounds with a 2-methylpropanoyl group were inactive, which is also indicating that a longer chain for a substituent at C-8 could be more favorable. In brief, from this SAR analysis, one may suggest that a geranyl-pyran group is more favorable than a geranyl-furan or a prenyl-furan substituent at C-5/C-6 of the 4-phenylcoumarin for enhancement of activity. Furthermore, a methylbutanoyl substituent is more favorable than a methylpropanoyl group for enhancement of the activity. Further chemical modifications of the natural compounds may still enhance the AChE inhibition activity, or may also reduce the activity of the compounds.

CHAPTER 6

NEUROPROTECTIVE EFFECTS OF 4-PHENYLCOUMARINS

A decade after stepping into the new millennium, neurodegenerative diseases still are one of the world's most arduous and appalling health issues as a result of increasing demographic fluctuation towards the aged population, as well as growing apprehension for a better quality of life. Such awareness, together with the huge social and economic costs of the disease, has ignited in depth research efforts to combat, or at least delay the onset of neurodegenerative diseases[104]. In a normal cycle of tissue proliferation, the aged and older cells are set to decease in order to give way for the generation of new cells. Such a setting of programmed cell death, or better known as apoptosis, is the prime cause in neurodegenerative diseases as a number of these diseases are characterized by a progressive fading away of neurons[105].

Apoptosis holds a cardinal role in the maturation of the nervous system, as well as neural architecture, through the interplay between both anti- and pro-apoptotic proteins. Basically, neurons either will render an adaptive response or they would initiate apoptosis when they are exposed to stress signals or apoptotic stimuli, such as withdrawal of neurotrophic factor, ischemic stroke, misfolded proteins, mitochondrial-complex inhibition, excessive calcium entry and excitotoxicity[106]. Apoptosis, or programmed cell death, is delineated with lucid and perspicuous morphological features, including cell shrinkage, chromatin condensation, loss of nuclear membrane integrity, plasma membrane blebbing or zeiosis, and eventually the breaking off of cellular fragments giving rise to the formation of apoptotic bodies[106]. Therefore, it is noteworthy that apoptosis orchestrates a crucial role in numerous neurodegenerative diseases, and targeting its pathway could confer a mode of prevention and treatment[105].

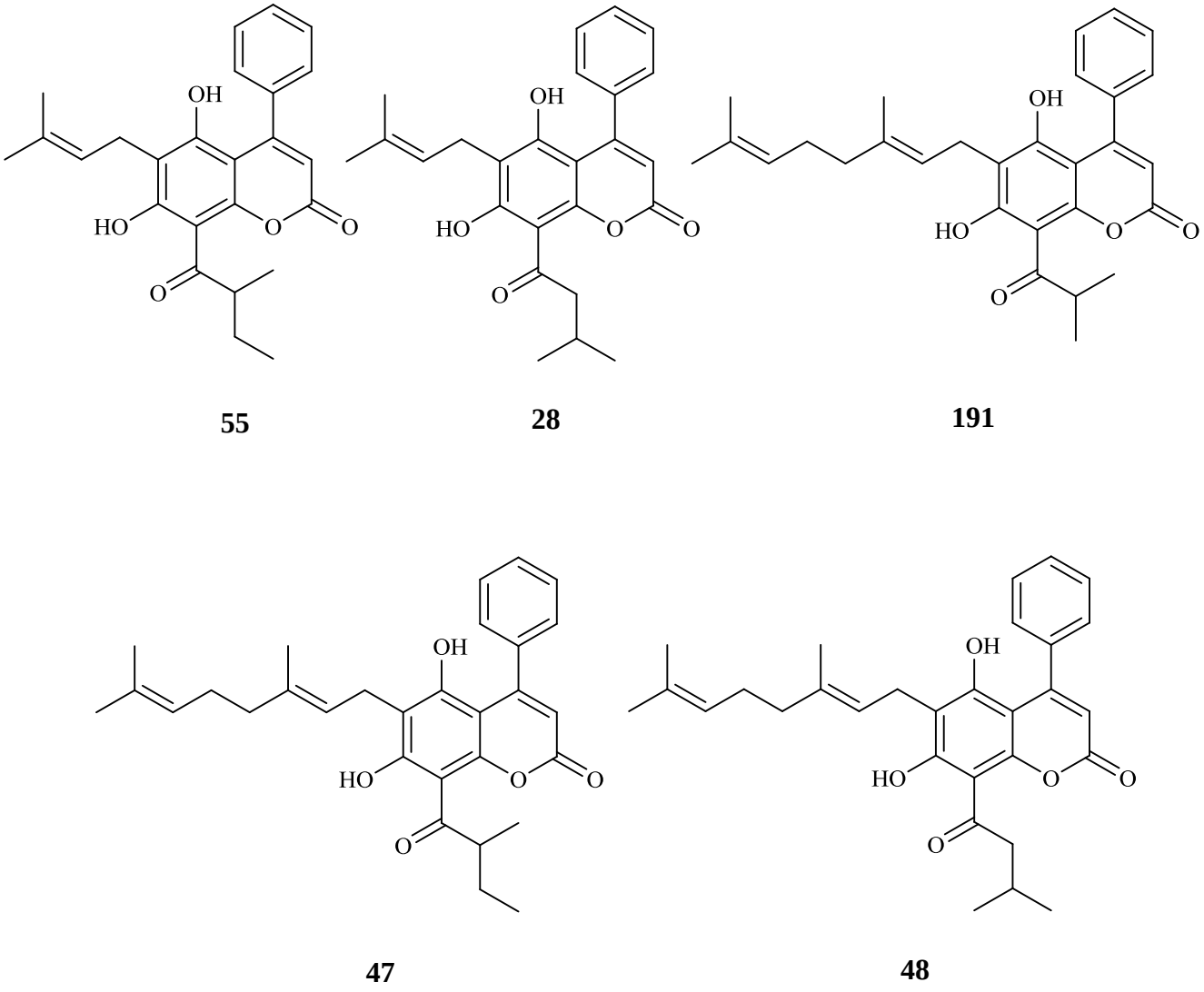
The present part of the work reports and discusses the neuroprotective effects of the extracts, fractions and pure compounds isolated from the bark of *Mesua kunstleri* (King) Kosterm, through a bioassay-guided evaluation.

6.1 Neuroprotective Activity Results

6.1.1 Bioassay-guided Study of the Neuroprotective Effects of *M. kunstleri*

The concentration-dependent effect of H_2O_2 challenge on cell viability for 10 hours using the MTT cell viability assay study was carried out[107]. NG108-15 cells were treated with different concentrations of H_2O_2 (0.1 - 4 mM) for 10 hours and a significant reduction in cell viability in a dose-dependent manner was detected. After exposure to 2 mM H_2O_2, NG108-15 cell viability was about 45.41 ± 3.42% that of control viability**.** Pretreatment (2 hours) with the hexane extract showed the highest percentage cell viability of 136.94 ± 2.03%, where as the ethyl acetate and methanol extracts showed 115.47 ± 3.55% and 89.81 ± 2.31%, respectively. The hexane extract was then subjected to neuroprotective assay-guided approach because of its high neuroprotective activity, and six fractions were obtained after fractionation. Fraction BH1 possessed the strongest neuroprotective activity with the percentage cell viability of 131.29 ± 3.03%. Further fractionation of BH1 yielded two sub-fractions (BH1-a and BH1-b) with sub-fraction BH1-b possessing stronger neuroprotective effect (101.07 ± 4.81%) as compared with fraction BH1-a. Hence, BH1-b was further chromatographed, and five pure compounds were isolated and identified. This neuroprotective assay-guided study has led to the isolation of five 4-phenylcoumarins from sub-fraction BH1-b, namely mammea A/BB **55**, mammea A/BA **28**, mesuagenin C **191**, 5,7-dihydroxy-8-(2-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one (DMDP-1) **47** and 5,7-dihydroxy-8-(3-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one (DMDP-2) **48** (Figure 6.1). Among these

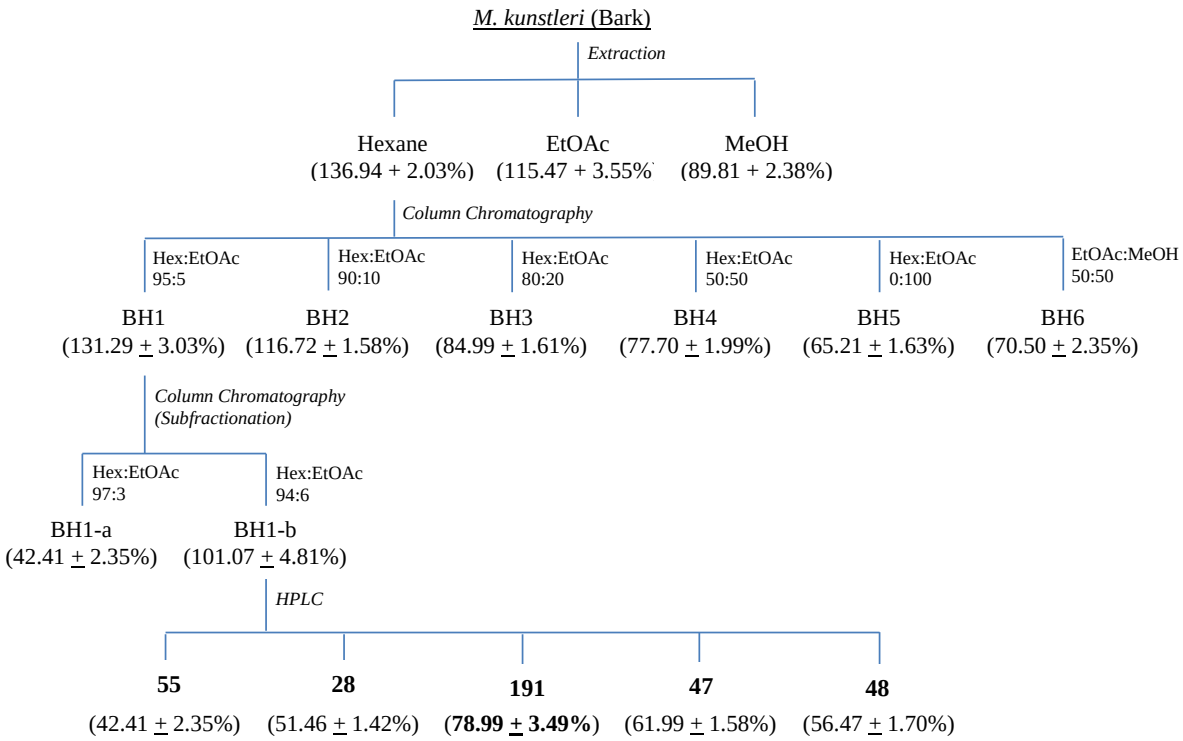
compounds, mesuagenin C **191** showed potent neuroprotective activity against H_2O_2 insult, and the results are depicted in Scheme 6.1.



55 **28** **191**

47 **48**

Figure 6.1: Compounds Isolated from Sub-fraction BH1-b from the Hexane Extract of *M. kunstleri*



Scheme 6.1: Fractionation and Isolation of the Bioactive Compounds from the Bark of *M. kunstleri* and Their Neuroprotective Effects at 200μM

6.1.2 Mesuagenin C Protective Effects Against H_2O_2-Induced Apoptosis in NG108-15 Cells

NG108-15 cells were pretreated with the 4-phenylcoumarins at varying concentrations (3.125 - 200 μM) for 2 hours prior to exposure to H_2O_2 (2 mM). As summarized in Table 6.1 and Figure 6.2 (a), only mesuagenin C **191** significantly increased the cell viability, up to 78.99 ± 3.49% (200 μM) which was the greatest neuroprotection as compared to the rest of the compounds. Furthermore, mesuagenin C **191** dose-dependently increased cell viability as shown in Figure 6.2 (b). These results allowed the conclusion that mesuagenin C **191** was effective for the protection of NG108-15 cells. EGCG was selected as a standard reference in the neuroprotective evaluation (Figure 6.2 (c)). Mesuagenin C **191** showed lower neuroprotective activity when

compared to EGCG at 50 μM (Figure 6.2 (c)). However, the neuroprotective activity of mesuagenin C **191** at concentrations of 25 μM and above were significantly different (*P < 0.05) compared to the H_2O_2-treated group.

Table 6.1: Neuroprotective Effects of 4-Phenylcoumarins (200 μM) Isolated from Hexane Extract of the Bark of *M. kunstleri* Against H_2O_2-Induced Apoptosis in NG108-15 cells.

Compound[a]	Cell Viability (%)[b]
Control	100
H_2O_2[c]	45.41 ± 3.42[#]
55	42.41 ± 2.35
28	51.46 ± 1.42[*]
191	78.99 ± 3.49[*]
47	61.99 ± 1.58[*]
48	56.47 ± 1.70[*]
EGCG (50 μM)[d]	85.19 ± 2.17

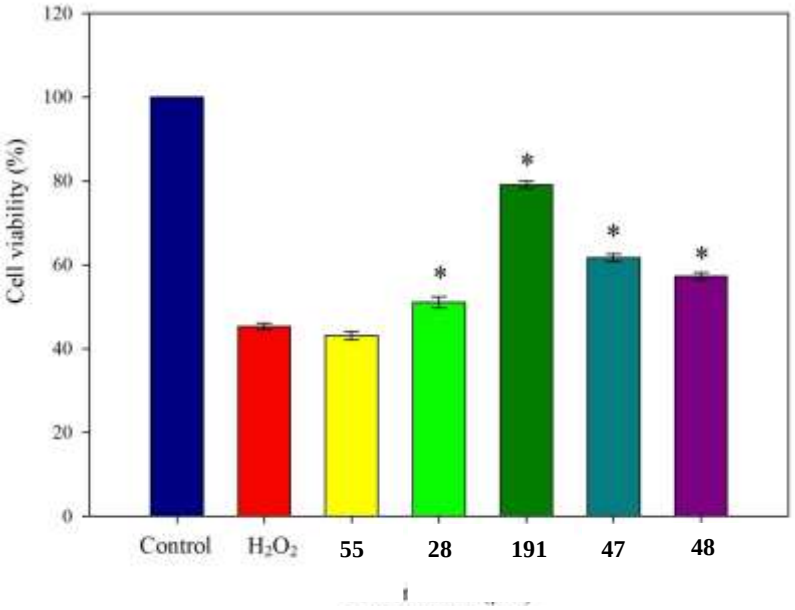
The values shown are the means ± SE of at least three independent experiments.

[a]NG108-15 cells were pretreated with 4-phenylcoumarins (3.125 - 200 μM) for 2 hours prior to exposure to H_2O_2 (2 mM). After incubation, cells were assessed by MTT to determine the percentage viability.
[b]Cell viability was measured by MTT assay.
[c]H_2O_2-treated value differed significantly from the untreated control at the level of #P < 0.05.
[d]EGCG was used as standard positive control at 50 μM.

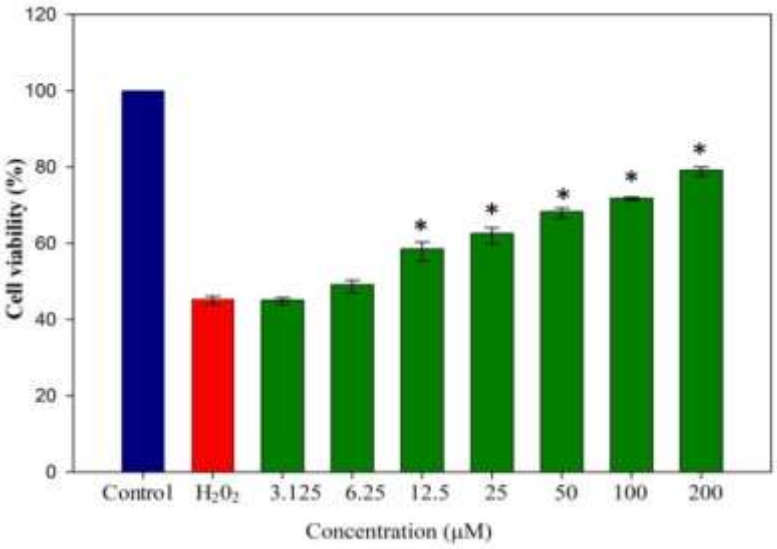
[#]Results differ significantly from H_2O_2-treated group compared to the control untreated group: P < 0.05.

[*]Significantly different from the H_2O_2-treated group compared to the treatment group: P < 0.05

(a)


Cell viability (%)
120
100
80
60
40
20
0
Control
H_2O_2
55
28
191
47
48
*

(b)


Cell viability (%)
120
100
80
60
40
20
0
Control
H_2O_2
3.125
6.25
12.5
25
50
100
200
Concentration (μM)
*

(c)

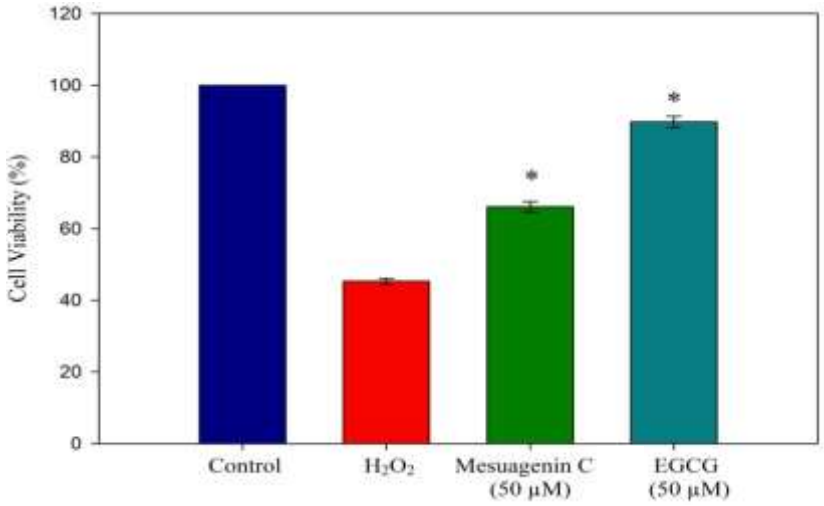


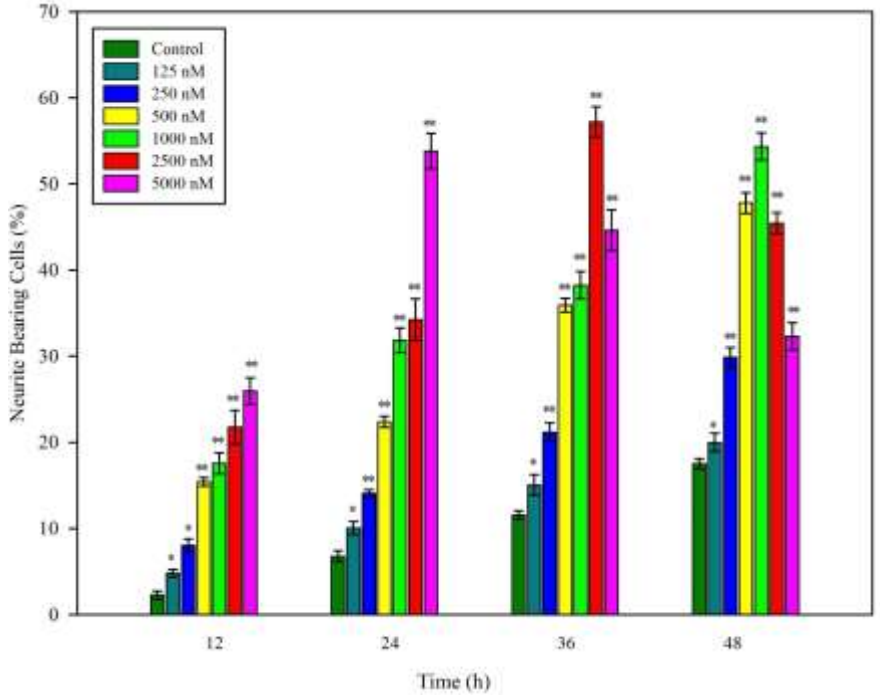
Figure 6.2: (a) Neuroprotective evaluation of 4-phenylcoumarins by MTT cell viability assay (b) Dose-dependent increase in cell viability by pretreatment with mesuagenin C **190** in H_2O_2-induced cell death prior to 2 mM of H_2O_2 exposure for 10 hours. Dose-dependent increase in cell viability by pretreatment with EGCG prior to 2 mM of H_2O_2 exposure for 10 hours. (c) Comparison of mesuagenin C **191** and EGCG pretreated cell viability. Values are mean ± S.E. from at least three independent experiments. The asterisk indicated significantly different values from H_2O_2-treated cells (*$P < 0.05$).

6.1.3 Effects of Mesuagenin C on Cell Viability and Neuritogenesis

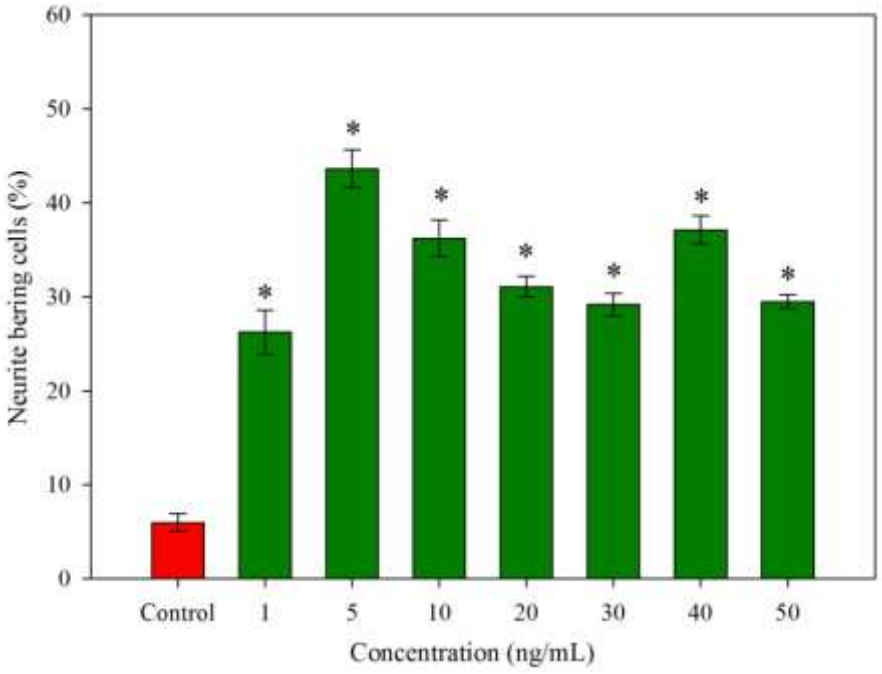
The effects of mesuagenin C **191** on neuritogenesis in NG108-15 cells were examined in both dose- and time-dependent experiments. The neuritogenic activity of mesuagenin C **191** was evaluated in the concentration range of 125 - 5000 nM for 12 - 48 hours of treatment. Following treatment with mesuagenin C **191** for 12 and 24 hours, the number of neurite-bearing cells was significantly increased in a dose-dependent manner, even at a lower concentration of 125 nM compared with untreated cells (Figure 6.3 (a)). As shown in Figure 6.3 (a), the number of neurite-bearing cells was rapidly promoted up to 53.83 ± 2.05% as compared to untreated cells ($P < 0.05$). Upon incubation for 36 hours at concentration of 2500 nM, the number of neurite-bearing cells was increased up to 57.22 ± 1.77%, which was the highest increment of neurite-bearing cells among the

different doses and time incubations. However, following incubation at 36 hours, treatment at 5000 nM recorded a decrease of the percentage of neurite-bearing cells (44.52 ± 2.48%), and this similar trend was observed when the cells were treated for 48 h at concentrations of 2500 and 5000 nM (Figure 6.3 (a)). Interestingly, it was observed that treatment with mesuagenin C **191** at 1000 nM significantly induced neuritogenesis in NG108-15 cells in both a dose- and time-dependent manner (Figure 6.3 (a)). When compared with time-matched, untreated cells and other concentrations, mesuagenin C **191** increased the number of neurite-bearing cells to 17.56, 31.85, 38.28 and 54.35% and based on this data, mesuagenin C **191** at a concentration of 1000 nM was used for the subsequent experiment. As compared to NGF-2.5S, a concentration of 5 ng/mL was found to be the optimal concentration that enhanced neurite outgrowth to 43.69 ± 1.78% as compared to the untreated group (Fig. 6.3 (b)). The evaluation of mesuagenin C **191** toxicity in NG108-15 was assessed by the MTT assay[107]. Mesuagenin C **191** at 100 and 200 nM increased NG108-15 cells proliferation with an increase of cell viability to 103.44% and 110.06%. Nevertheless, the initial toxicity was seen at 3.125 µM at 48 hour (Figure 6.3 (c)). Even though the cell viability was reduced to 50% with mesuagenin C **191** concentration of (IC_{50}) 20.09 ± 1.58 µM, thus reassuring the concentration of mesuagenin C **191** (125-5000 nM) was safe, non-toxic and 20-fold lower (at 1000 nM) than its IC_{50} value in the neuritogenesis analysis.

(a)


Control
125 nM
250 nM
500 nM
1000 nM
2500 nM
5000 nM
Neurite Bearing Cells (%)
Time (h)
12
24
36
48

(b)


Neurite bering cells (%)
Concentration (ng/mL)
Control
1
5
10
20
30
40
50

(c)

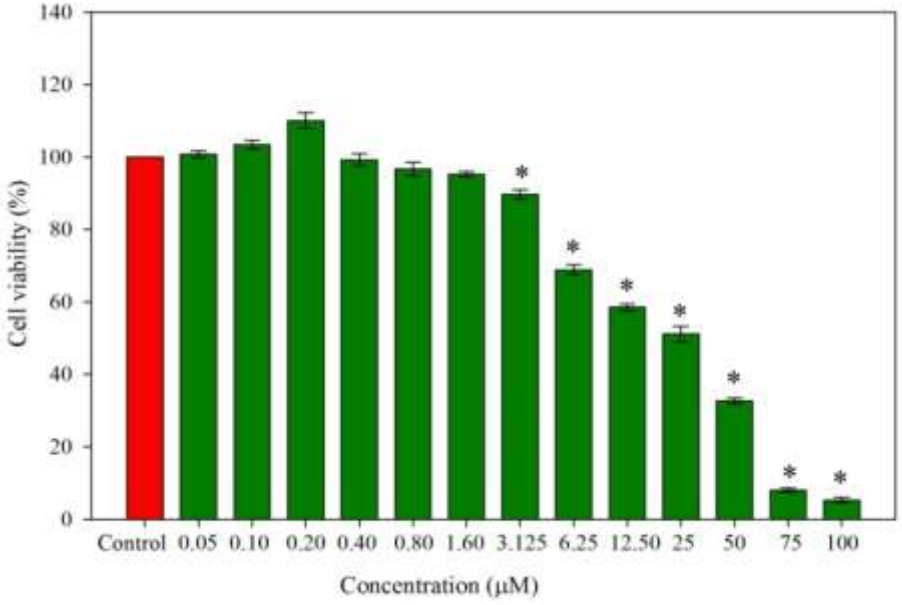


Figure 6.3: Mesuagenin C **191** induced neuritogenesis in NG108-15 cells and its effects on NG108-15 cell viability. (a) NG108-15 cells were treated with mesuagenin C **191** for 12, 24, 36 and 48 hours, analysed by phase-contrast microscopic analysis. Mesuagenin C **191** significantly induced neurite outgrowth in a dose-dependent manner (125-5000 nM) after 12 - 48 hours. (b) The effects of NGF-2.5S on neurite outgrowth in NG108-15 cells. NGF-2.5S was used as the positive control. (c) The cytotoxicity of mesuagenin C **191** after 48 hours of treatment in NG108-15 cells. Values are mean ± S.E. from at least three independent experiments. The asterisk (*P < 0.05 and **P < 0.01) indicated significantly different values from untreated cells.

6.2 Neuroprotective Effects of 4-Phenylcoumarins and their SAR studies

In the present study, it was found that the hexane fraction of *M. kunstleri* prevented H_2O_2-induced neurotoxicity in NG108-15 cells. In order to clarify the neuroprotective components of *M. kunstleri*, as part of a continuing study on neuroprotective effects of *M. kunstleri*, neuroprotective activity-guided isolation was carried out to search for the active fractions and compounds. The effects of various extracts on neuroprotective activity were compared and the crude hexane extract of *M. kunstleri* exerted the most potent neuroprotective activity against H_2O_2-induced toxicity in NG108-15 cells (Scheme 6.1). The activity of the crude hexane extract is 136.94 ± 2.03% of cell viability, which is higher than the activity of the most active fraction from this crude (BH1), which has 131.29 ± 3.03% of cell viability. Further sub-fractionation of the most active fraction gave rise to BH1-b being the most active sub-fraction with 101.07 ± 4.81% of cell viability. The neuroprotective assay-guided fractionation and isolation of the active chemical constituent led to the identification of five compounds from the most potent sub-fraction BH1-b; mammea A/BB **55**, mammea A/BA **28**, mesuagenin C **191**, 5,7-dihydroxy-8-(2-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one (DMDP-1) **47** and 5,7-dihydroxy-8-(3-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one (DMDP-2) **48**.

Isolated compounds from this subfraction gave rise to mesuagenin C **191** with the highest activity (78.99 ± 3.49% of cell viability). The activity values of the cell viability evaluation declined from the crude hexane extract to the pure isolated compounds, which led to the hypothesis of the involvement of a synergistic mechanism. All of the five compounds isolated in sub-fraction BH1-b possess an open chain prenylated or geranylated 4-phenylcoumarin skeleton. Based on the MTT assay, mesuagenin C **191** illustrated the most significant neuroprotective activity towards H_2O_2-induced cell death

in the NG108-15 cells. These results suggested that the C-6 side chain of the 4-phenylcoumarin is essential for the neuroprotective activity. Compounds possessing a free geranyl chain at position C-6 (**191**, **47** and **48**) are active whereas compounds possessing free prenyl chain at the same position (**55** and **28**) are less active. Further isolation work was done on subfraction BH1-a, and cyclized prenylated (mammea A/BB cyclo D **186** and mammea A/BA cyclo D **187**) and geranylated 4-phenylcoumarins (mesuagenin E **188**, mesuagenin A **189** and mesuagenin B **190**) were purified and characterized (Fig. 6.4). These compounds were isolated from the less active subfraction BH1-a, which suggests that a cyclized prenyl and geranyl groups at position C-6 of the 4-phenylcoumarins are not essential for the activity.

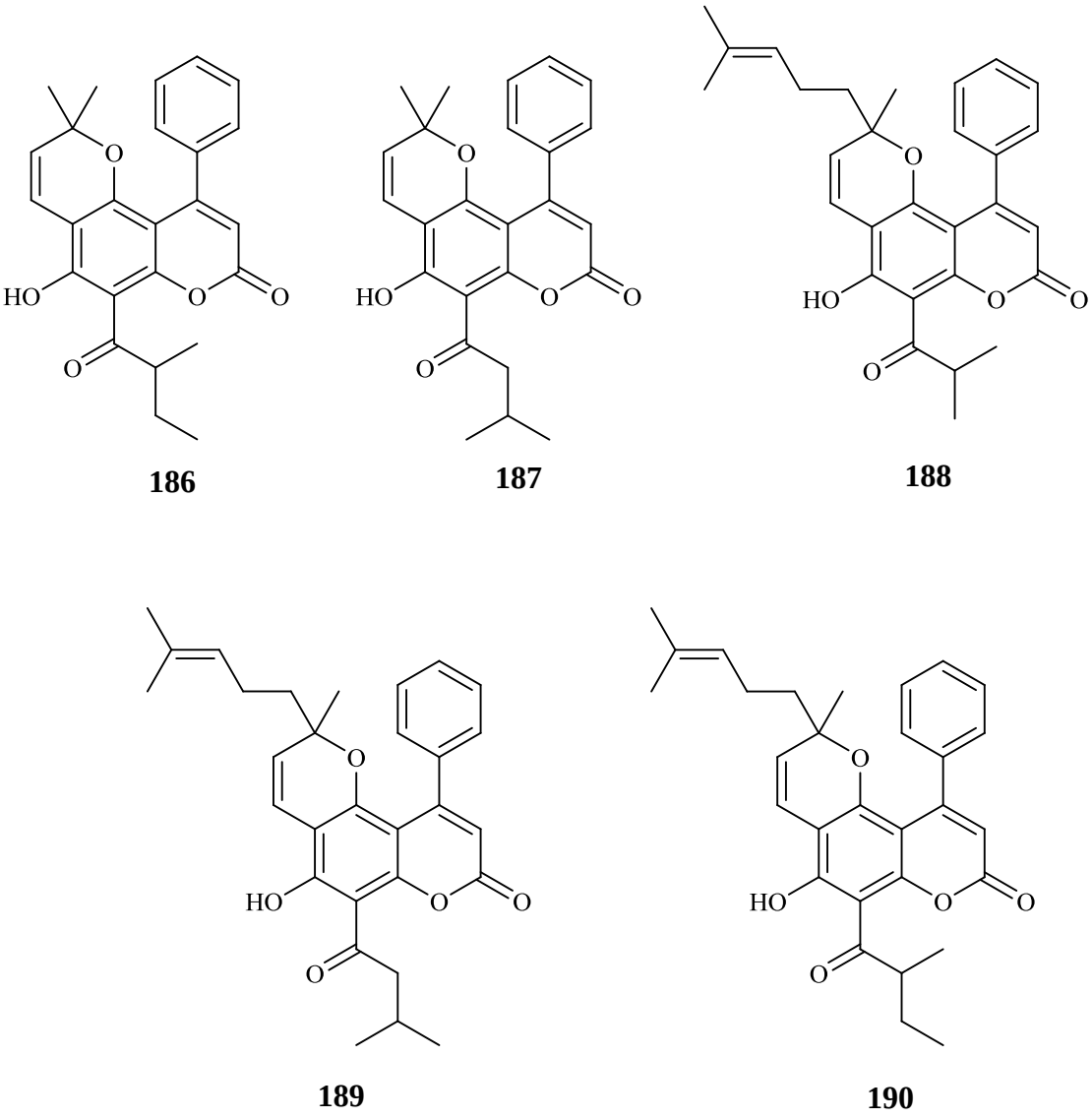


Figure 6.4: Compounds Isolated from Sub-fraction BH1-a from the Hexane Extract of *M. kunstleri*

CHAPTER 7

CONCLUSIONS

Phytochemical analysis of the bark of two Malaysian Clusiaceae plants, *Mesua elegans* (King) Kosterm. and *Mesua kunstleri* (King) Kosterm., were carried out. A total of twenty-two compounds were isolated from *M. elegans*, of which thirteen are new; mesuagenin A **189**, mesuagenin B **190**, mesuagenin C **191**, mesuagenin F **195**, mesuagenin D **196**, mesuagenin G **198**, mesuagenin H **199**, mesuagenin I **200**, mesuagenin J **201**, mesuagenin K **202**, mesuagenin L **203**, mesuagenin M **204** and mesuagenin N **205**. From *M. kunstleri*, a total of ten compounds were isolated, of which one is new; mesuagenin E **188**. These compounds were purified using several chromatographic techniques; column chromatography (CC), thin layer chromatography (TLC), preparative thin layer chromatography (PTLC), centrifugal partition chromatography (CPC) and high performance liquid chromatography (HPLC). The structures of the isolated compounds were elucidated using various spectroscopic methods; 1D NMR (^{1}H, ^{13}C and DEPT), 2D NMR (COSY, HSQC, HMBC and NOESY), UV, IR, HRESIMS, and by comparison with data from literature.

Preliminary screening of the bark from *M. elegans* showed that the hexane extract possessed acetyl cholinesterase (AChE) inhibitory activity with the inhibition percentage of 90% at 10 μg/mL. Bioassay-guided study of the active hexane extract afforded mesuagenin A **189**, mesuagenin B **190**, 5,7-dihydroxy-8-(2-methylbutanoyl)-6-[(*E*)-3,7-dimethylocta-2,6-dienyl]-4-phenyl-2*H*-chromen-2-one **47** and mesuagenin D **196** as good inhibitors of AChE, especially mesuagenin B **190** with an IC_{50} of 0.70 μM. Further isolation work carried out with this hexane extract afforded more natural 4-phenylcoumarins, and semi-synthesis work was done on the major 4-

phenylcoumarins from this extract, which afforded seventeen semi-synthesized 4-phenylcoumarins. All of the natural and semi-synthetic 4-phenylcoumarin analogues were subjected to an AChE inhibition SAR study. From this SAR analysis, it is suggested that a geranyl-pyran group is more favorable than a geranyl-furan or a prenyl-furan substituent at C-5/C-6 of the 4-phenylcoumarin for enhancement of activity. Furthermore, a methylbutanoyl substituent is more favorable than a methylpropanoyl group for enhancement of the activity. Further chemical modifications of the natural compounds may enhance the AChE inhibition activity further, or, on the contrary, may also reduce the activity of the compounds.

Bioassay-guided study was also carried out to isolate and evaluate the neuroprotective compounds from the hexane extract of the bark of *M. kunstleri* on H_2O_2-induced apoptosis in NG108-15 cells. Mesuagenin C **191** was isolated as the most active compound with 78.99 ± 3.49% of cell viability after NG108-15 cells were exposed to 2 mM H_2O_2 and 2 hours of pretreatment with this compound. From the cell viability evaluation of the ten 4-phenylcoumarins (from sub-fraction BH1-a and BH1-b), it may be suggested that compounds possessing a free geranyl chain at position C-6 are more favourable for the neuroprotective activity than compounds possessing a free prenyl chain at the same position. It may also be proposed that a cyclized prenyl or geranyl group at position C-6 of the 4-phenylcoumarins is not essential for the activity.

This study has shown that *Mesua* plants are rich producers of interesting bioactive compounds of the 4-phenylcoumarin type. Further studies can be performed on other *Mesua* species, and for the potent compounds, elucidation of their mechanism of actions may be investigated. 4-Phenylcoumarins may be potential lead compounds for further investigation as therapeutic agents for neurodegenerative diseases.

CHAPTER 8

EXPERIMENTAL

8.1 Plant Materials

Mesua elegans

The bark of *Mesua elegans* (King) Kosterm. was collected from Sungai Badak Forest Reserve, Sintok, Kedah, Malaysia on 27th April 2006 by Mr. Din bin Muhammad Nor. The sample (Figure 8.1) with voucher specimen number KL 5232, was identified by Mr. Teo Leong Eng and deposited in the herbarium of the Department of Chemistry, Faculty of Science, University of Malaya.

Figure 8.1: *Mesua elegans* (King) Kosterm.

Plant description:

The tree *Mesua elegans* (King) Kosterm. was 8 m tall and 15 cm in diameter. The bark was greyish brown and scaly with pinkish brown inner bark. The leaves were thinly coriaceous, from elliptic to lanceolate with apex acuminate acute and base acute to obtuse. The areas of the leaves are 6 - 10.5 cm x 2 - 3.5 cm with dark green above and glaucous below. The leaves have 16 - 20 pairs of nerves, which were faint and visible on both sides. The petioles were short, with the length of 3 - 5 mm long. The tree had inflorescence axillary and terminal, which were solitary or cymose. The flowers of the tree are white in colour.

Mesua kunstleri

The bark of *Mesua kunstleri* (King) Kosterm. was collected from Rimba Teloi Forest Reserve, Kedah, Malaysia on 19th April 1995 by Mr. Din bin Muhammad Nor. The sample (Figure 8.2) with voucher specimen number KL 4485 was identified by Mr. Teo Leong Eng and deposited in the herbarium of the Department of Chemistry, Faculty of Science, University of Malaya.

Figure 8.2: *Mesua kunstleri* (King) Kosterm.

Plant description:

The tree *Mesua kunstleri* (King) Kosterm. was 14 m tall and 35 cm in diameter. The bark was grey and with pinkish red inner barks and brown exudate. The leaves were opposite simple, thinly coriaceous and in distant pairs, from elliptic to lanceolate with long acuminate acute and base shortly pointed. The areas of the leaves are 9.5 - 13 cm x 2.5 - 4 cm with bright green above and slightly glaucous below. The leaves have prominent 20 pairs of nerves. The petioles were 7 mm in length. The tree had inflorescence axillary and terminal, which were solitary and sometimes several were together. The flowers of the tree are yellow-green in colour.

8.2 Instrumentation

- NMR spectra were obtained using JEOL LA400 FT NMR, JEOL ECA400 FT NMR Spectrometer System, Bruker AVN400 FT NMR, Bruker AVN600 FT NMR, Bruker DMX500 WB and Bruker 300 NMR using deuterated chloroform as solvent. Chemical shifts were reported in ppm and coupling constants were given in Hertz (Hz).
- Mass spectra were carried out on an Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS, with ZORBAX Eclipse XDB-C18 Rapid Resolution HT 4.6 mm i.d. x 50 mm x 1.8 μm column. HPLC grade methanol, acetonitrile and deionised water were used as mobile phase solvents. All solvents and samples were filtered with 0.2 μm nylon membrane filter (WHATMAN) prior to LCMS analysis.
- UV spectra were recorded on a Shimadzu UV-Visible Recording Spectrophotometer using HPLC grade ethanol as solvent with mirror UV cell.
- The infrared (IR) spectra were obtained through Perkin Elmer FT-IR Spectrometer Spectrum RX1 using chloroform as solvent.

8.3 Chromatography

8.3.1 Thin Layer Chromatography (TLC)

Aluminum supported silica gel 60 F_{254} plates were used to visualize the spots of the isolated compounds. UV Light Model UVGL-58 Mineralight Lamp 230V~50/60 Hz was used to examine spots or bands on the TLC after spraying with the required reagents.

8.3.2 Column Chromatography (CC)

All solvents used in these experiments were industrial grade (distilled). Silica gel 60, 230-400 mesh ASTM (Merck 9385) was used for column chromatography. A slurry of silica gel 60 (approximately 30:1 silica gel to sample ratio) in hexane solvent system was poured into a glass column of appropriate size with gentle tapping to remove trapped air bubbles. The crude extract was initially dissolved in a minimum amount of solvent and loaded on top the packed column. The extract was eluted with an appropriate solvent system at a certain flow rate. Fractions were collected in either test tubes or conical flasks and evaporated for the next step. Fractions with similar compounds were then combined after TLC monitoring.

8.3.3 Preparative Thin Layer Chromatography (PTLC)

PTLC silica gel 60 F_{254} glass plates of size 20 cm x 20 cm (Merck 1.05715.0001) were used for the separation of compounds. UV Light Model UVGL-58 Mineralight Lamp 230V~50/60 Hz was used to examine bands on the PTLC.

8.3.4 High Performance Liquid Chromatography (HPLC)

Waters HPLC System was used for HPLC separation, equipped with Binary Gradient Module, System Fluidics Organizer and UV detector set at the range from 200-600 nm. HPLC injections were also performed with a Rheodyne 20 µL sample loop (Rheodyne Europe, Bensheim, Germany) associated with a Hewlett-Packard series 1100 HPLC degasser G1322A, QuatPump G13211A and DAD G1315A (Agilent Technologies, Massy, France). Chromatographic analysis and separations were performed on ZORBAX Eclipse Plus C18 (4.6 mm i.d. x 150 mm x 3.5 µm), ZORBAX Eclipse Plus C18 (9.4 mm i.d. x 250 mm x 3.5 µm), Chromolith Performance RP-18 (4.6 mm i.d. x 100 mm), Chromolith RP-18 endcapped (10 mm i.d. x 100 mm), Genesis C18 (4.6 mm

i.d. x 150 mm x 4.0 μm), Genesis C18 (10 mm i.d. x 150 mm x 4.0 μm) and Purospher C18 (4.6 mm i.d. x 250 mm x 5 μm) HPLC columns. Methanol (HPLC grade), acetonitrile (HPLC grade) and deionized water were used as mobile phase solvents with formic acid (HPLC grade) as buffer. All solvents and samples were filtered with 0.45 μm nylon membrane filter prior to HPLC analysis. The data were collected and analysed by using MassLynx software.

8.3.5 Centrifugal Partition Chromatography (CPC)

Armen Instruments CPC was used for CPC separations; SCPC-250 system coupled with Armen Glider Spot Prep II CPC software and provided by Armen Technologies (France). The SCPC-250 system was equipped with a 250 mL column with injection capacity ranging from 10 mg to 3-6 g. The rotation speed was adjustable from 0 to 3000 rpm. Binary gradient pump with a flow-rate that ranges from 5 to 15 mL/min, built-in automatic injection valve, UV-vis dual wavelength spectrophotometer with high pressure flow cell were also used. The rotor was first filled with the upper phase of the solvent system as the stationary phase. The apparatus was rotated at 1600 rpm and the lower mobile phase of the solvent mixture was then pumped into the inlet of the column (rotor) at a flow rate of 10 mL/min in the descending mode. Samples were loaded in the 5 mL injection-loop, and injected in the column in a "sandwich" mode, i.e. at the same time with the mobile phase. First elution occurred in the descending mode (reversed-phase mode): the rotor was filled with the upper apolar phase of the solvent mixture, and the pumped mobile phase is the polar lower phase. The first batch was collected in descending mode for 50, 150, 300 and 500 mg of samples. After collecting fractions in the descending mode by peak, another batch was injected to collect as multidual mode, where by the switching valve is turned to the ascending mode and the mobile phase pumped is the upper one this time. The descending-ascending switching went on until

all the peaks observed in the descending mode were eluted. Extrusion was performed after the last tube; the lower phase was pumped in the ascending mode to eject the sum of the upper phase out of the rotor[108,109]. *n*-Heptane (Hept), ethyl acetate (EtOAc), methanol (MeOH), acetonitrile (CH_3CN) were purchased from Carlo Erba Reactifs (Val de Reuil, France) as analytical grade solvents used for CPC analyses. Trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich (Lyon, France). Water was of ultrapure quality. Samples were filtered with 0.45 μm nylon membrane filter prior to injection.

8.4 Detection Reagents

8.4.1 Vanillin-Sulphuric Acid

Vanillin (1.0 g) in concentrated H_2SO_4 (10 mL) was added upon cooling to 90 mL of ethanol before spraying onto the TLC plate. The TLC plate was then heated at ~50 ºC until full development of the colours had occurred. The occurrence of blue, purple, dark green, grey or brown spots indicated the presence of coumarins and phenylpropenes.

8.4.2 Anisaldehyde-Sulphuric Acid

Anisaldehyde (0.5 mL) was added in glacial acetic acid (50 mL) and 1 mL concentrated H_2SO_4. After the reagent was sprayed onto the TLC plate, the TLC plate was then heated to ~105°C until maximum visualization of spots had occurred. The occurrence of purple, blue, red, grey or green spots indicated the presence of phenols, sugars, steroids, and terpenes.

8.5 Extraction and Isolation

8.5.1 Extraction and Isolation of *Mesua elegans*

The extraction, fractionation and isolation of the bark of *Mesua elegans* (King) Kosterm. were performed using the bioassay-guided study method, in which after each

stage, the sample was tested for the activity; in this case for the acetyl cholinesterase (AChE) inhibitory activity.

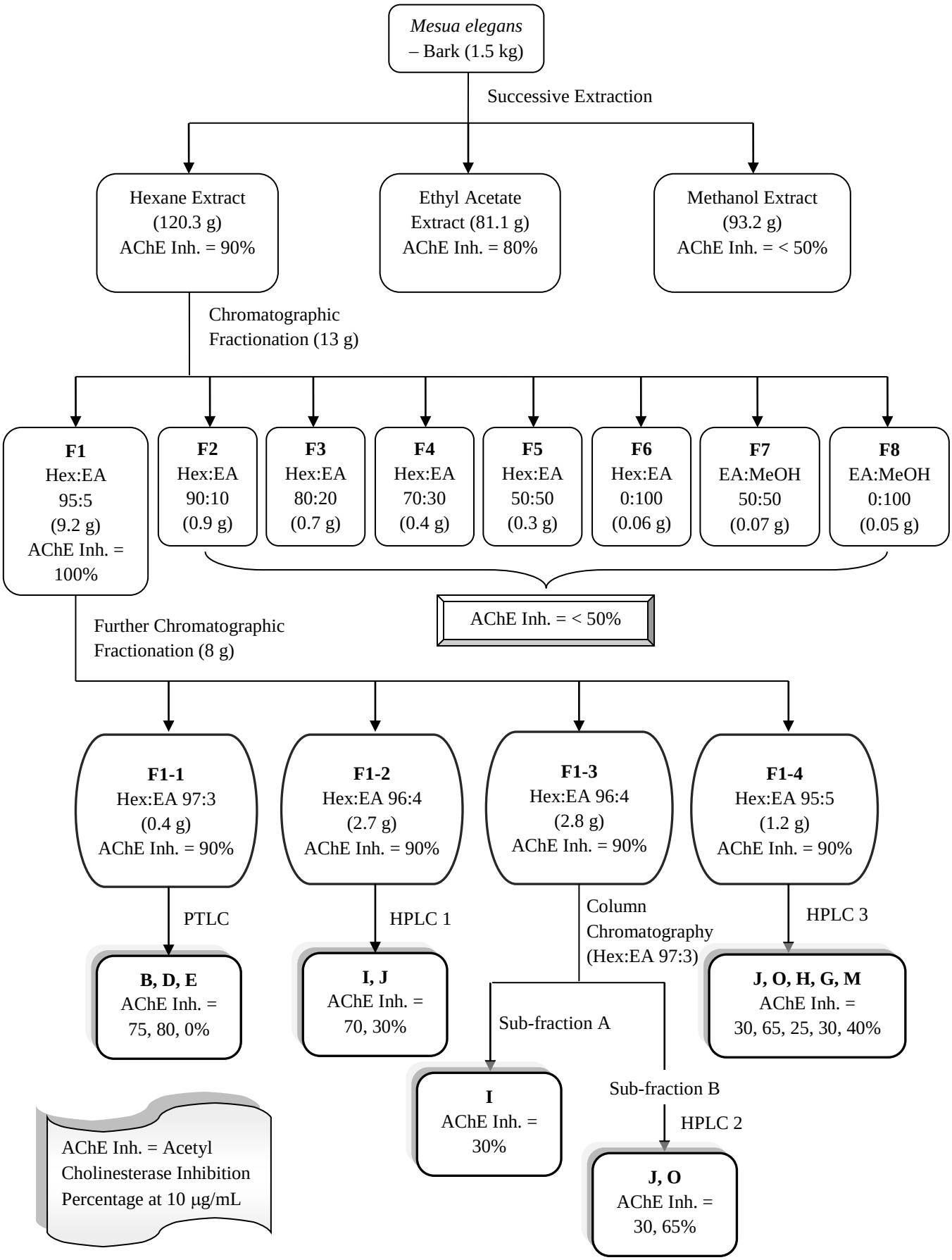
The extraction was carried out by the cold percolation method. Dried ground bark (1.5 kg) was macerated with hexane (3 x 4L, each 48 hours) at room temperature. The hexane extract was then evaporated using rotary-evaporator and a yellow gummy extract (120.3 g) was obtained. The plant material was then subjected to ethyl acetate and methanol extraction successively (3 x 4L, each 48 hours, respectively) at room temperature. The extracts were then evaporated using a rotary-evaporator. The ethyl acetate crude (81.1 g) was obtained as a brown gummy residue while the methanol extract (93.2 g) was obtained as a brown amorphous powder. All the crude extracts were tested for AChE inhibitory activity, and the hexane extract gave the highest inhibitory activity (90% inhibition at 10 μg/mL concentration). Thus, the hexane extract was subjected to further chromatographic analysis. Both the crude ethyl acetate and methanol extracts were kept for future use and study.

The crude hexane extract of the bark (13.0 g) was subjected to column chromatography fractionation over silica gel (230-400 mesh). The amount of eluent solvent used was based on the ratio of 1 g of crude extract to 1 L of solvent for each fraction. Thus, in this case, 13 L of eluent solvent was used for each fraction. All the fractions were then tested for AChE inhibitory activity. Fraction 1 (F1) was the most potent fraction with 100% inhibition at 10 μg/mL concentration. Thus F1 was subjected for further isolation and purification steps.

F1 (8.0 g) was subjected to column chromatography over silica gel. The further fractionation steps were based on the gradient elution method. Sub-fractions were

collected for every 500 mL of eluent. The sub-fractions were then treated individually and subjected to either further column chromatography (CC), Preparative Thin Layer Chromatography (PTLC), High Performance Liquid Chromatography (HPLC) or Centrifugal Partition Chromatography (CPC) analysis. The bioassay-guided isolation procedure of AChE inhibitors from the bark of *Mesua elegans* are outlined in Scheme 8.1. Listed in Table 8.1 are the PTLC conditions for the purification of F1-1. HPLC conditions for F1-2, F1-3-sub-fraction B and F1-4 are shown in the next few pages, along with their respective HPLC profiles in Figures 8.3 - 8.5. Scheme 8.2 shows the isolation procedure of fraction F2, where as Scheme 8.3 and Scheme 8.4 show the isolation procedure for fraction F3 and F4, respectively.

Structural identification of the isolated compounds was carried out using spectroscopic methods; ^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC, DEPT, IR, UV and mass spectrometry. Compounds isolated from the bark of *M. elegans* and their yield, are listed in Table 8.2.



Scheme 8.1: Bioassay-Guided Isolation Procedure of AChE Inhibitors from the Bark of *Mesua elegans* (King) Kosterm.

Table 8.1: PTLC Condition for the Purification of F1-1

Condition	Compound D **189**	Compound E **190**	Compound B **187**
Solvent System	Hexane : Ethyl Acetate 98 : 2; developed 4 times		
R_f (Hex:EA _ 98:2)	0.26	0.26	0.21

HPLC 1 (F1-2)

LC mode : Analytical

LC column : ZORBAX Eclipse Plus C18 (4.6 x 150 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-20 min, 50-100% B

Flow Rate : 1 mL/min

Weight of sample injected : 20 mg

Sample injection volume : 10 μL

Sample injection concentration : 1 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound I **47** - 18.81 min

Compound J **48** - 19.05 min

HPLC 2 (F1-3-Sub-fraction B)

LC mode : Semi-preparative

LC column : Genesis C18 (10.0 x 150 mm, 4.0 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: ACN + Formic Acid 0.1%; 0-30 min, 60-100% B

Flow Rate : 4.7 mL/min

Weight of sample injected : 120 mg

Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound O **196** - 18.60 min

Compound J **48** - 24.22 min

HPLC 3 (F1-4)

LC mode : Semi-preparative

LC column : Chromolith RP18 Endcapped (10.0 x 100 mm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-20 min, 50-100% B; 20-25 min, 100-100% B

Flow Rate : 4.7 mL/min

Weight of sample injected : 150 mg

Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound M **194** - 12.00 min

Compound G **28** - 17.45 min

Compound O **196** - 18.81 min

Compound H **191** - 20.87 min

Compound J **48** - 22.22 min

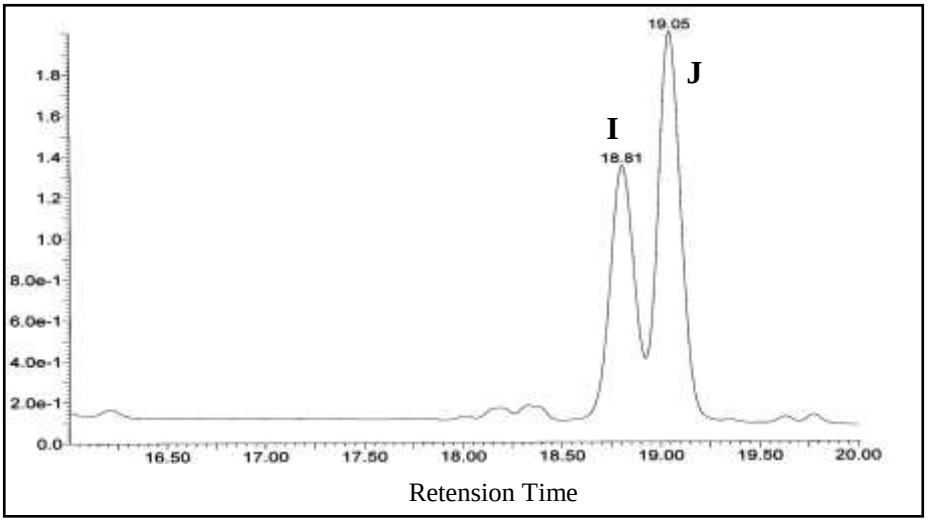


Figure 8.3: HPLC Profile of Fraction F1-2 (HPLC 1)

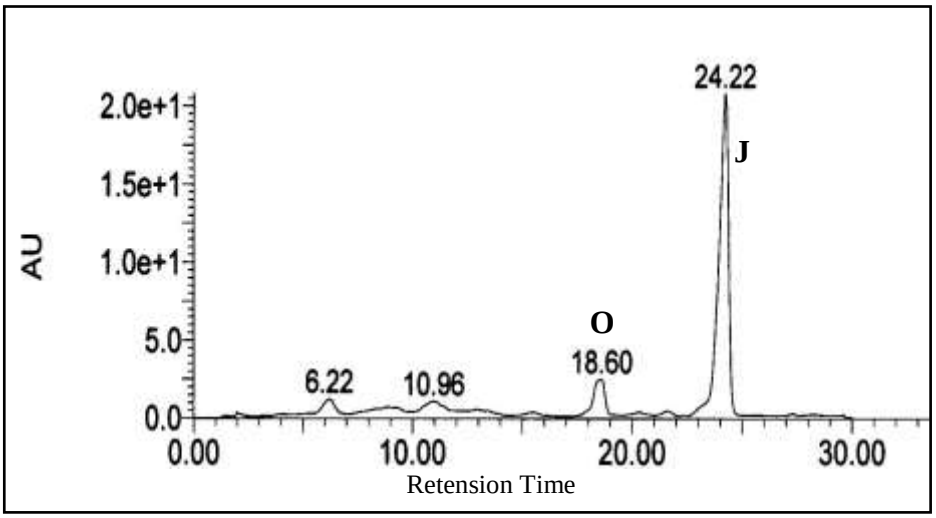


Figure 8.4: HPLC Profile of F1-3-Sub-fraction B (HPLC 2)

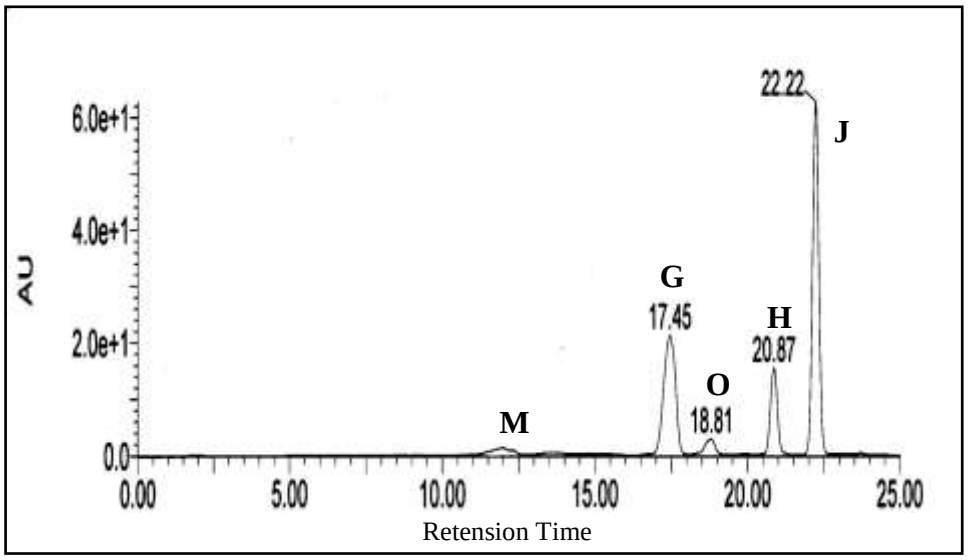
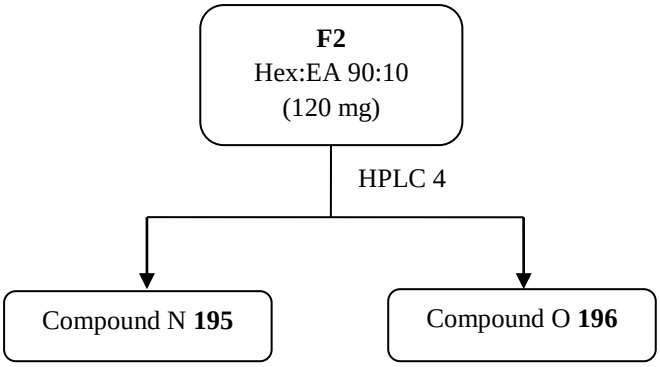


Figure 8.5: HPLC Profile of Fraction F1-4 (HPLC 3)



Scheme 8.2: Isolation Procedure of F2 from the Bark of *Mesua elegans*

HPLC 4 (F2)

LC mode : Semi-preparative

LC column : ZORBAX Eclipse Plus C18 (9.4 x 250 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-60 min, 85-85% B; 60-61 min, 85-100% B; 61-67 min, 100-100% B; 67-68 min, 100-85% B; 68-75 min, 85-85% B

Flow Rate : 3 mL/min

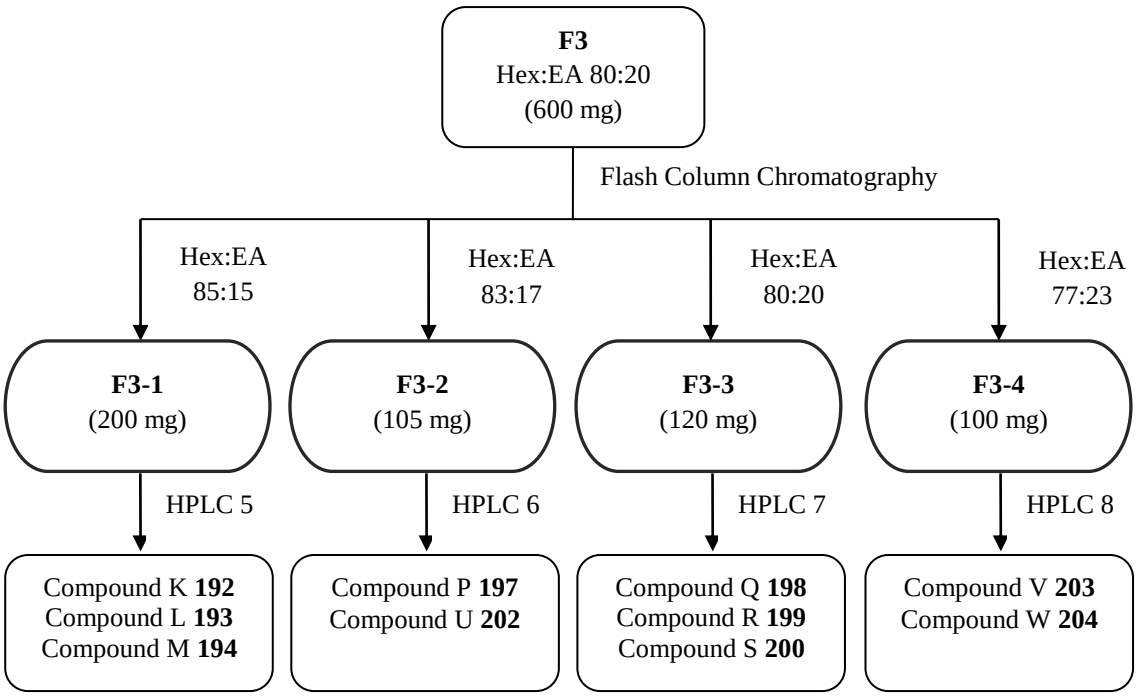
Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound N **195** – 41.47 min

Compound O **196** – 47.29 min



Scheme 8.3: Isolation Procedure of F3 from the Bark of *Mesua elegans*

HPLC 5 (F3-1)

LC mode : Semi-preparative

LC column : ZORBAX Eclipse Plus C18 (9.4 x 250 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-80 min, 85-85% B; 80-81 min, 85-100% B; 81-90 min, 100-100% B; 90-91 min, 100-85% B; 91-100 min, 85-85% B

Flow Rate : 3 mL/min

Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound K **192** - 43.75 min

Compound L **193** - 56.72 min

Compound M **194** - 73.25 min

HPLC 6 (F3-2)

LC mode : Semi-preparative

LC column : ZORBAX Eclipse Plus C18 (9.4 x 250 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-110 min, 82-82% B; 110-120 min, 82-100% B; 120-130 min, 100-100% B; 130-131 min, 100-82% B; 131-140 min, 82-82% B

Flow Rate : 3 mL/min

Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound P **197** - 29.41 min

Compound U **202** - 98.12 min

HPLC 7 (F3-3)

LC mode : Semi-preparative

LC column : ZORBAX Eclipse Plus C18 (9.4 x 250 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-100 min, 85-85% B; 100-101 min, 85-100% B; 101-110 min, 100-100% B; 110-111 min, 100-85% B; 111-120 min, 85-85% B

Flow Rate : 3 mL/min

Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound Q **198** - 67.59 min

Compound R **199** - 79.63 min

Compound S **200** - 85.90 min

HPLC 8 (F3-4)

LC mode : Semi-preparative

LC column : ZORBAX Eclipse Plus C18 (9.4 x 250 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-55 min, 80-80% B; 55-56 min, 80-100% B; 56-63 min, 100-100% B; 63-64 min, 100-80% B; 64-70 min, 80-80% B

Flow Rate : 3 mL/min

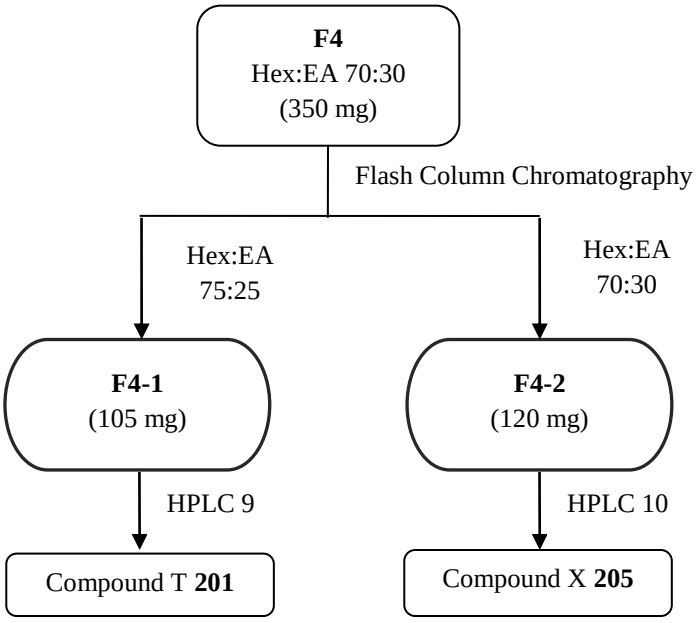
Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound V **203** - 49.57 min

Compound W **204** - 52.30 min



Scheme 8.4: Isolation Procedure of F4 from the Bark of *Mesua elegans*

HPLC 9 (F4-1)

LC mode : Semi-preparative

LC column : ZORBAX Eclipse Plus C18 (9.4 x 250 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-40 min, 75-75% B; 40-60 min, 75-100% B; 60-70 min, 100-100% B; 70-71 min, 100-75% B; 71-80 min, 75-75% B

Flow Rate : 3 mL/min

Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound T **201** - 31.95 min

HPLC 10 (F4-2)

LC mode : Semi-preparative

LC column : ZORBAX Eclipse Plus C18 (9.4 x 250 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-45 min, 80-80% B; 45-46 min, 80-100% B; 46-52 min, 100-100% B; 52-53 min, 100-80% B; 53-60 min, 80-80% B

Flow Rate : 3 mL/min

Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound X **205** - 27.66 min

Table 8.2: Chemical Constituents from *M. elegans* and Their Yield

Isolates	**Yield (mg)**
Compound B	40.3
Compound D	55.1
Compound E	15.1
Compound F	5.2
Compound G	7.3
Compound H	20.0
Compound I	3.2 / 30.2 (CPC)
Compound J	5.6 / 56.3 (CPC)
Compound K	5.0
Compound L	7.3
Compound M	15.1
Compound N	35.2
Compound O	60.3
Compound P	3.1
Compound Q	5.0
Compound R	6.5
Compound S	7.5
Compound T	11.3
Compound U	4.0
Compound V	3.0
Compound W	2.5
Compound X	12.4

8.5.1.1 CPC Separation of *Mesua elegans*

A CPC study was carried on with the fraction F1 (hexane: ethyl acetae 95:5) of *Mesua elegans*. Comparison study between isocratic mode and multiple dual-mode of CPC injections have been carried out for 50, 150, 300 and 500 mg injections of fraction F1. Apart from CPC modes comparison, both the CPC modes (isocratic and multiple dual-mode) have been compared with semi-preparative HPLC separations as well. Five 4-phenylcoumarins have been identified in these investigations; compound F **55**, compound G **28**, compound H **191**, compound I **47** and compound J **48**. From the CPC separations, in both isocratic mode and multiple dual-mode, fraction II contained a mixture of compounds F **55** and G **28**, fraction III contained compound G **28**, fraction IV contained compound H **191** and fraction V contained a mixture of compounds I **47** and J **48**.

Two 4-phenylcoumarins were isolated as pure compounds by using CPC in both isocratic and multiple-dual mode injections; compound G **28** and compound H **191**. The purity of the CPC isolated compounds G **28** and H **191** were shown in Table 8.3. In the isocratic mode, only 50 and 150 mg injections gave compounds G **28** with a HPLC (293 nm) purity of above 90%; 50 mg (98.5%) and 150 mg (96.2%). Increment of the injected mass to 300 and 500 mg in the isocratic mode did not manage to give compounds G **28** with a purity above 90% (Figure 8.6). Meanwhile in the multiple dual-mode, all the injection gave compounds G **28** with a purity of above 90%; 50 mg (99.6%), 150 mg (97.6%), 300 mg (93.2%) and 500 mg (93.0%). For compound H **191**, the CPC separation in the isocratic mode for the 50, 150 and 300 mg injections gave a HPLC (293 nm) purity of above 90%; 50 mg (98.1%), 150 mg (94.6%) and 300 mg (91.1%) (Figure 8.7). As for the multiple dual-mode separation, all the injections gave a purity of above 90% for compound H **191**; 50 mg (100%), 150 mg (96.6%), 300 mg

(95.5%) and 500 mg (94.3%). Until 150 mg injection, differences of HPLC purity for both compounds between isocratic and multiple-dual modes were insignificant. The chromatogram for the comparison between 150 and 300 mg multiple-dual modes were depicted in Figure 8.8. However, when injected masses were higher, the purity decreased below 90% in isocratic mode, where as in multiple-dual mode, compound G **28** and compound H **191** were up to 93% pure even when 500 mg of sample were injected. From the purity results of compound G **28** and compound H **191**, it may be deduced that CPC multiple-dual mode separation was better than isocratic mode separation for these compounds.

Another comparative study of the percentage yield between semi-preparative HPLC and CPC separations (both isocratic and multiple dual-modes) has also been conducted. Table 8.4 displayed the yield and percentage recovery of fraction H1 using both semi-preparative HPLC and CPC separations techniques. Recovery of total mass injected from semi-preparative HPLC separation was merely 85.0%, whereas for the CPC separations (both modes), gave a recovery with more than 90%. Yields obtained in 150, 300 and 500 mg injections from CPC isocratic and multiple dual-modes were also compared, in which 300 mg gave the highest yield; 94.8% in batch mode, and 93.7% in multiple-dual mode.

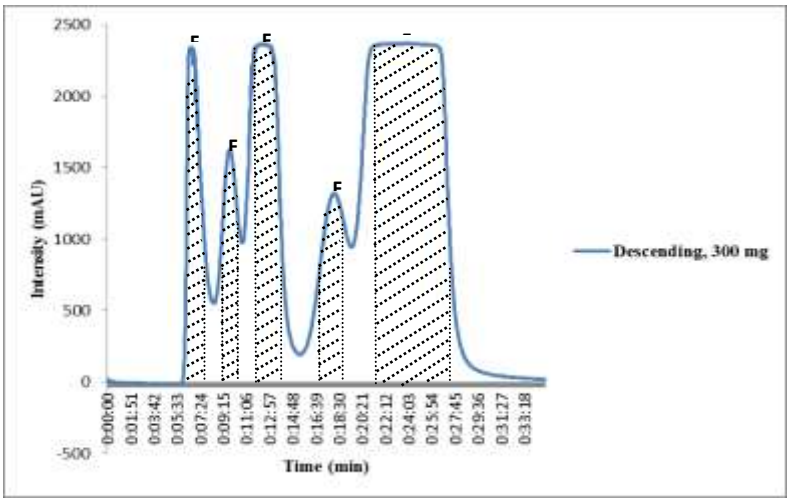


Figure 8.6: CPC chromatogram of 300 mg injection, isocratic mode. Column capacity: 250 mL, solvent system: heptane-ethyl acetate-acetonitrile-water (9:1:9:1, v/v/v/v), containing trifluoroacetic acid 0.1%, mobile phase: lower phase, flow rate: 10 mL/min, rotation speed of column: 1600 rpm, retention of stationary phase: 72.8%.

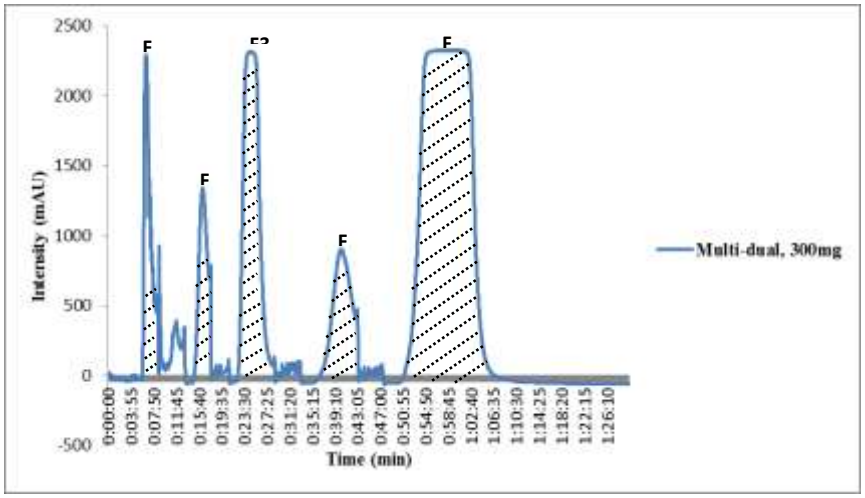


Figure 8.7: CPC chromatogram of 300 mg injection, multiple dual-mode. Column capacity: 250 mL, solvent system: heptane-ethyl acetate-acetonitrile-water (9:1:9:1, v/v/v/v), containing trifluoroacetic acid 0.1%, mobile phase: lower phase in descending mode and upper phase in ascending mode, flow rate: 10 mL/min, rotation speed of column: 1600 rpm, retention of stationary phase in descending mode: 72.8%.

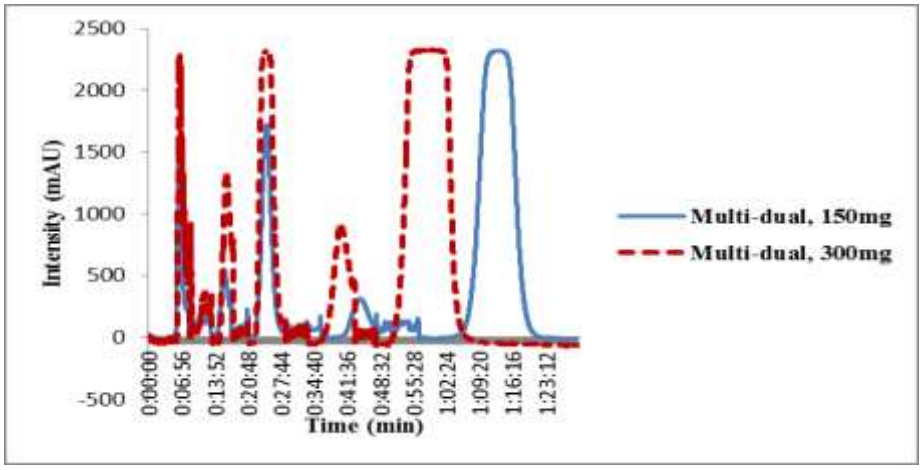


Figure 8.8: Comparison between CPC chromatograms of multiple dual-mode 150 mg and 300 mg injections. Column capacity: 250 mL, solvent system: heptane-ethyl acetate-acetonitrile-water (9:1:9:1, v/v/v/v), containing trifluoroacetic acid 0.1%, mobile phase: lower phase in descending mode and upper phase in ascending mode, flow rate: 10 mL/min, rotation speed of column: 1600 rpm, retention of stationary phase in descending mode: 72.8%.

Table 8.3: HPLC Purity Comparison of Compound G **28** and Compound H **191** at 293 nm Isolated by Isocratic and Multiple-Dual Mode CPC Injections

Mode	Isocratic		Multiple dual	
Mass Injected (mg)	Compound G **28** (% purity)	Compound H **191** (% purity)	Compound G **28** (% purity)	Compound H **191** (% purity)
50	98.5	98.1	99.6	100.0
150	96.2	94.6	97.6	95.5
300	84.2	91.1	93.0	96.6
500	82.3	79.4	93.2	94.3

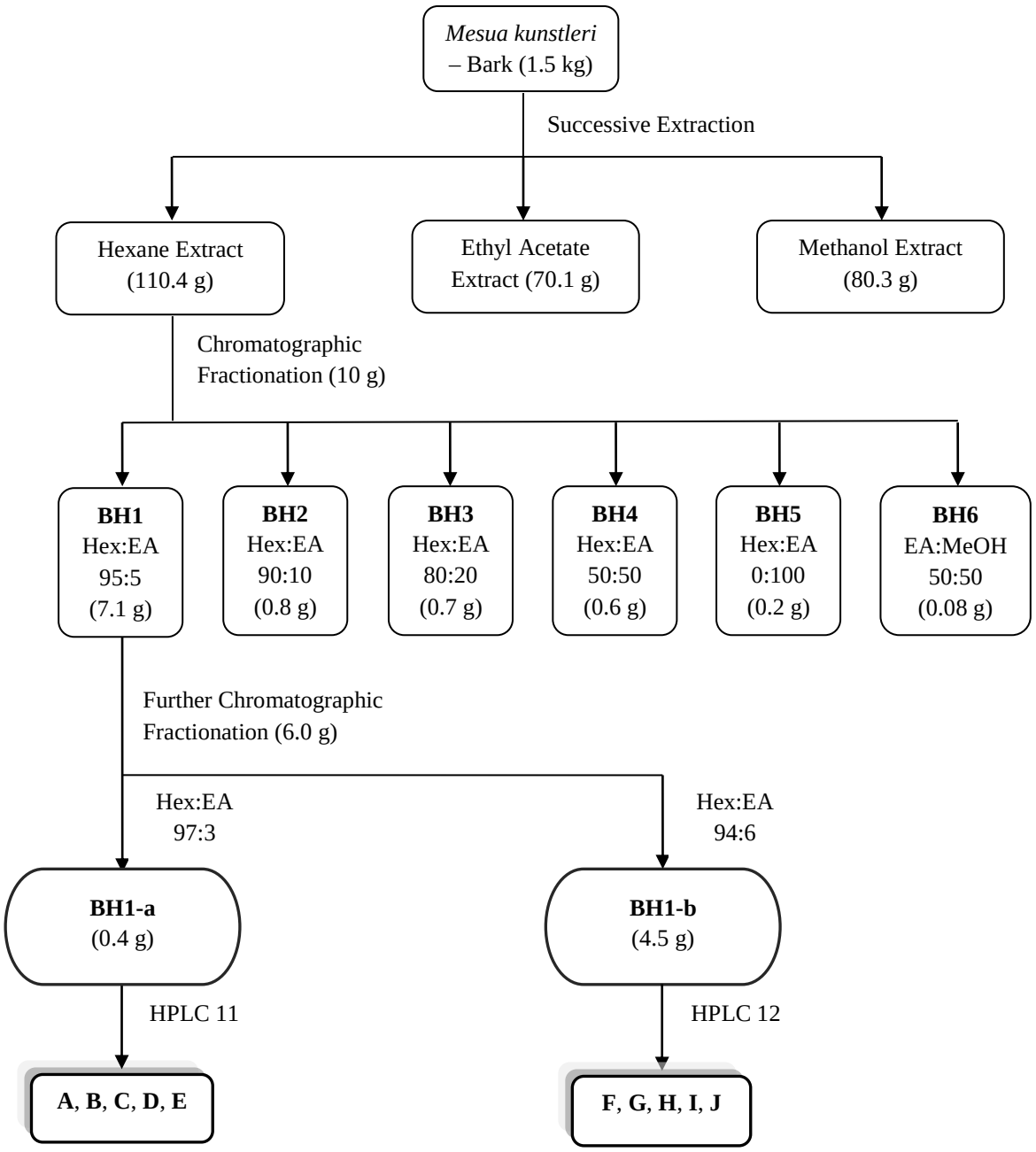
Table 8.4: Yield and Percentage Recovery Comparison Between HPLC and CPC (Isocratic and Multiple-Dual Mode) Injections of Fraction F1

Mode	Semi-Preparative HPLC	Isocratic CPC				Multiple dual CPC			
Fractions (mg)	10	50	150	300	500	50	150	300	500
Total Mass of Recovery (mg)	8.5	45.5	139.8	284.3	451.0	46.1	139.5	281.0	463.1
% Recovery	85.0	91.0	93.2	94.8	90.2	92.2	93.0	93.7	92.6

8.5.2 Extraction and Isolation of *Mesua kunstleri*

Dried ground bark of *M. kunstleri* (1.5 kg) was macerated with hexane (3 x 4L, each 48 hours) at room temperature. The hexane extract was dried off using rotary-evaporator and some yellow gummy residue (110.4 g) were obtained. The plant material was then subjected for ethyl acetate and methanol extraction successively (3 x 4L, each 48 hours, respectively) at room temperature. Both of the extracts were then evaporated off to dryness using rotary-evaporator. The ethyl acetate crude (70.1 g) was obtained as brown gummy residue while the methanol extract (80.3 g) was obtained as brown amorphous powder. Hexane crude exhibited the most potent neuroprotection activity (136.94 $\pm$ 2.03%). Thus, the hexane crude was subjected for further chromatographic analysis. Both ethyl acetate and methanol crude were kept for future use and study.

A portion of the hexane crude (10.0 g) was subjected to column chromatography fractionation over silica gel (230-400 mesh) and eluted with hexane-EtOAc (from 9.5 to 0) and EtOAc-MeOH (5:5) to give fractions BH1-BH6. Fraction BH1 showed the most potent neuroprotective activity (131.29 $\pm$ 3.03%), thus fraction BH1 was then subjected to silica gel chromatography and eluted with hexane-EtOAc (from 9.7 to 9.4) to produce subfractions BH1-a and BH1-b. Both of these subfractions were subjected for neuroprotective assay study and BH1-b was found to be the most active subfraction (101.07 $\pm$ 4.81%). The isolation procedure of the neuroprotective compounds from the bark of *M. kunstleri* are outlined in Scheme 8.5. Compounds isolated from the bark of *M. kunstleri* and their yield, are listed in Table 8.5.



Scheme 8.5: Isolation Procedure of Chemical Constituents from the Bark of *Mesua kunstleri* (King) Kosterm.

HPLC 11 (BH1-a)

LC mode : Semi-preparative

LC column : ZORBAX Eclipse Plus C18 (9.4 x 250 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-180 min, 85-85% B; 180-181 min, 85-100% B; 181-190 min, 100-100% B; 190-191 min, 100-85% B; 191-200 min, 85-85% B

Flow Rate : 3 mL/min

Weight of sample injected : 150 mg

Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound A **186** - 48.14 min

Compound B **187** - 52.60 min

Compound C **188** - 105.99 min

Compound D **189** - 157.20 min

Compound E **190** - 174.74 min

HPLC 12 (BH1-b)

LC mode : Semi-preparative

LC column : ZORBAX Eclipse Plus C18 (9.4 x 250 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-50 min, 90-90% B; 50-51 min, 90-100% B; 51-60 min, 100-100% B; 60-61 min, 100-90% B; 61-70 min, 90-90% B

Flow Rate : 3 mL/min

Weight of sample injected : 200 mg

Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound F **55** - 17.15 min

Compound G **28** - 17.89 min

Compound H **191** - 31.26 min

Compound I **47** - 42.09 min

Compound J **48** - 44.05 min

Table 8.5: Chemical Constituents from *M. kunstleri* and Their Yield

Isolates	**Yield (mg)**
Compound A	3.2
Compound B	5.2
Compound C	8.0
Compound D	20.2
Compound E	30.3
Compound F	8.1
Compound G	15.3
Compound H	10.6
Compound I	30.5
Compound J	50.8

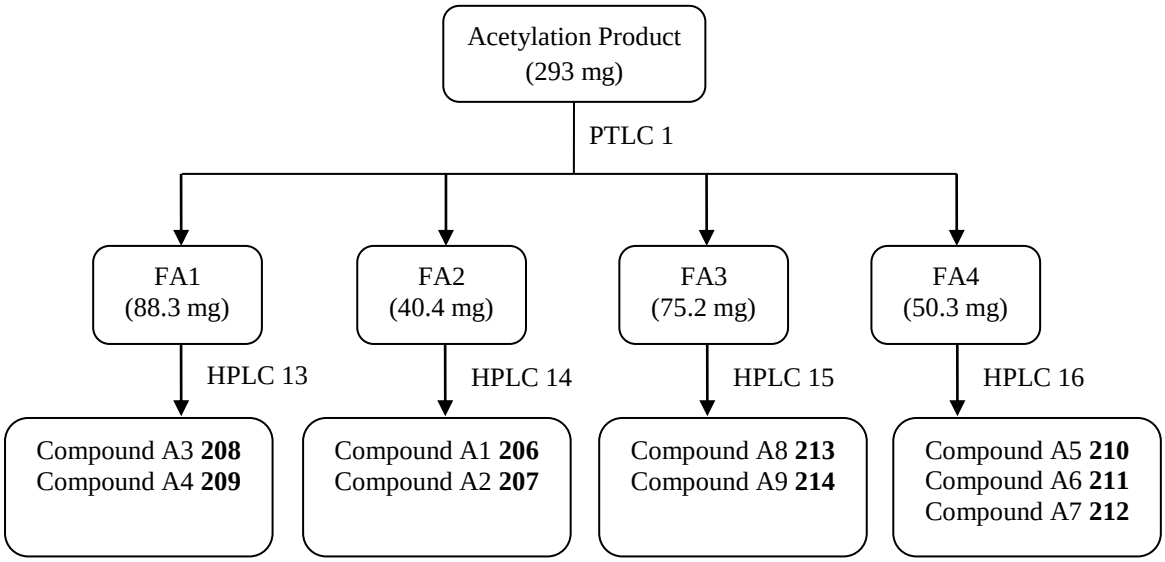
8.6 Semi-synthesis

8.6.1 Acetylation

Fraction F1 (hexane:ethyl acetate 95:5) from the hexane extract of the bark of *Mesua elegans* was subjected to the acetylation process. This fraction mainly contained five chemical constituents; compound F, compound G, compound H, compound I and compound J. Below is the method used for the acetylation. The semi-synthesized compounds were isolated from the acetylation product (Scheme 8.6) and the following Table 8.6 shows the PTLC condition for the fractionation of acetylation products. Table 8.7 shows the names of the isolates and yield obtained.

The method

Acetic anhydride (4.3 mL, 45.47 mmol) was added to a solution of 305 mg of fraction F1 in pyridine (5.0 mL). After stirring for 14 hours, the solution was concentrated with vacuum evaporator and chromatographed using PTLC and HPLC[110].



Scheme 8.6: Isolation Procedure of the Acetylation Product

Table 8.6: PTLC Condition for the Fractionation of Acetylation Product

Condition	FA1	FA2	FA3	FA4
Solvent System	Hexane : Ethyl Acetate 90 : 10; developed 3 times			
R_f Hex:EA 90:10	0.90	0.84	0.75	0.60

HPLC 13 (FA1)

LC mode : Semi-preparative

LC column : ZORBAX Eclipse Plus C18 (9.4 x 250 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-150 min, 95-95% B; 150-151 min, 95-100% B; 151-160 min, 100-100% B; 160-161 min, 100-95% B; 161-170 min, 95-95% B

Flow Rate : 3 mL/min

Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound A4 **209** - 132.98 min

Compound A3 **208** - 150.39 min

HPLC 14 (FA2)

LC mode : Semi-preparative

LC column : ZORBAX Eclipse Plus C18 (9.4 x 250 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-40 min, 85-85% B; 40-41 min, 85-100% B; 41-50 min, 100-100% B; 50-51 min, 100-85% B; 51-60 min, 85-85% B

Flow Rate : 3 mL/min

Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound A9 **214** - 30.15 min

Compound A8 **213** - 34.89 min

HPLC 15 (FA3)

LC mode : Semi-preparative

LC column : ZORBAX Eclipse Plus C18 (9.4 x 250 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-30 min, 80-80% B; 30-31 min, 80-100% B; 31-40 min, 100-100% B; 40-41 min, 100-80% B; 41-50 min, 85-80% B

Flow Rate : 3 mL/min

Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound A6 **211** – 11.60 min

Compound A5 **210** – 12.79 min

Compound A7 **212** – 23.20 min

HPLC 16 (FA4)

LC mode : Semi-preparative

LC column : ZORBAX Eclipse Plus C18 (9.4 x 250 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-50 min, 85-85% B; 50-51 min, 85-100% B; 51-60 min, 100-100% B; 60-61 min, 100-85% B; 61-70 min, 85-85% B

Flow Rate : 3 mL/min

Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound A2 **207** – 39.87 min

Compound A1 **206** – 44.13 min

Table 8.7: Acetylation Isolates and Their Yield

Acetylation Isolates	Yield (mg)
Compound A1 **206**	12.5
Compound A2 **207**	19.8
Compound A3 **208**	21.6
Compound A4 **209**	35.6
Compound A5 **210**	12.3
Compound A6 **211**	17.3
Compound A7 **212**	11.3
Compound A8 **213**	19.5
Compound A9 **214**	30.2

8.6.2 Methylation

Methylation was carried out using fraction F1 (hexane:ethyl acetate 95:5) from the hexane extract of the bark of *Mesua elegans*. The parent compounds F - J in this fraction were methylated to afford compounds M1 - M4. Table 4.2 depicts the isolates of the methylation process.

The method

A mixture of fraction F1 (499.3 mg), potassium carbonate (583.1 mg, 4.21 mmol) and iodomethane (449.0, 3.16 mmol) in dry acetone was heated to reflux over 8 hrs. The

mixture was then be filtered to remove potassium carbonate and was concentrated. The residue were dissolved in ethyl acetate and washed with water. The organic layer were dried over sodium sulphate, evaporated at reduced pressure and chromatographed by HPLC (HPLC 17). The methylation product gained was 450.3 mg. Table 8.8 shows the names of the isolates and yield obtained.

HPLC 17 (Methylation Product)

LC mode : Semi-preparative

LC column : ZORBAX Eclipse Plus C18 (9.4 x 250 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-100 min, 85-85% B; 100-101 min, 85-100% B; 101-110 min, 100-100% B; 110-111 min, 100-85% B; 111-120 min, 85-85% B

Flow Rate : 3 mL/min

Weight of sample injected : 160 mg

Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound M1 **215** - 26.43 min

Compound M2 **216** - 58.56 min

Compound M4 **218** - 80.57 min

Compound M3 **217** - 85.39 min

Table 8.8: Methylation Isolates and Their Yield

Methylation Isolates	Yield (mg)
Compound M1 **215**	15.6
Compound M2 **216**	24.3
Compound M3 **217**	33.6
Compound M4 **218**	56.9

8.6.3 Bromobenzoylation

Fraction F1-2 from the hexane extract of the bark of *Mesua elegans* was been subjected to the bromobenzoylation process. This fraction contained two major chemical constituents; compound I **47** and compound J **48**. Table 8.9 designates the isolates from the bromobenzoylation reaction.

The method

To a solution fraction F1-2 (500.0 mg) and dry acetone (20 mL), potassium carbonate (437.0 mg, 3.16 mmol) and benzylbromide (541.1 mg, 3.16 mmol) were added. The mixture was heated for reflux for 12 hrs. The solution was then diluted with ethyl acetate, and was washed with brine and water, respectively. The organic phase were dried over sodium sulphate, evaporated at reduced pressure and chromatographed by HPLC (HPLC 18). The bromobenzoylation product gained was 443.3 mg. Table 8.9 shows the names of the isolates and yield obtained.

HPLC 18 (Bromobenzoylation Product)

LC mode : Semi-preparative

LC column : ZORBAX Eclipse Plus C18 (9.4 x 250 mm, 3.5 μm)

Mobile system: A: H_2O + Formic Acid 0.1%; B: MeOH + Formic Acid 0.1%; 0-105 min, 90-90% B; 105-106 min, 90-100% B; 106-115 min, 100-100% B; 115-116 min, 100-90% B; 116-125 min, 90-90% B

Flow Rate : 3 mL/min

Weight of sample injected : 150 mg

Sample injection volume : 500 μL

Sample injection concentration : 10 mg/mL

UV detection (nm) : 200-400, PDA detector

Retention time of compound : Compound B1 **219** - 46.27 min

Compound B2 **220** - 52.89 min

Compound B3 **221** - 88.20 min

Compound B4 **222** - 98.11 min

Table 8.9: Bromobenzoylation Isolates and Their Yield

Bromobenzoylation Isolates	Yield (mg)
Compound B1	10.2
Compound B2	15.3
Compound B3	30.2
Compound B4	53.6

8.7 Acetyl Cholinesterase Inhibitory Activity Evaluation

Acetylcholinesterase (AChE) from *Electrophorus electricus* (C2888) was purchased from Sigma. Inhibition of AChE activity was determined by the spectroscopic method of Ellman, using acetylthiocholine iodide as substrate, in 96-well microtiter plates. All solutions were brought to room temperature prior to use. Aliquots of 200 μL of a solution containing 640 μL of 10 mM of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) in 0.1M sodium phosphate, pH 8.0, 19.2 mL of the same buffer, and 13 μL of a solution of AChE (100 U/mL) in water, were added to each well, followed by 2 μL of a DMSO solution of the inhibitor (0.9% final volume). The reaction was initiated by adding 20 μL of acetylthiocholine iodide (7.5 mM) to each well, and was followed by monitoring the appearance of the thiolate dianion produced by the reduction of DTNB at 412 nm every 13 s for 120 s at 25 °C in a Molecular Devices spectra Max 384 Plus plate reader. Each inhibitor was evaluated at ten concentrations (ranging from 1 mg/mL to 0.05

μg/mL by diluting by a factor of 3). Percentage inhibition was calculated relative to a control sample of DMSO (% inhibition = [1-(slope cpd/slope DMSO)] x 100) with SoftMax® Pro software. IC_{50} values displayed represent the mean ± standard deviation for six assays ($SD = [\sum(x - x)^2/n]^{1/2}$). Tacrine hydrochloride (Sigma, purity > 99%) was used as reference compound[111].

8.8 Neuroprotective Activity Evaluation

NG108-15 hybridoma cell line was obtained from American Type Culture Collection (ATCC). Dulbecco's Modified Eagle's Medium (DMEM), epigallocatechin gallate (EGCG), Hypoxanthine-Aminopterin-Thymidine (HAT), phosphate buffered saline (PBS), sodium bicarbonate, HEPES salt and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were provided by Sigma-Aldrich. Fetal bovine serum (FBS), 100 unit/mL penicillin/100 μg/mL streptomycin and amphotericin B (50 μg/mL) were supplied by PAA Laboratories. Accutase was bought from Innovative Cell Technologies, Inc. Hydrogen peroxide (H_2O_2) was purchased from Systerm®. Murine Nerve Growth Factor 2.5s was purchased from Promega. All chemicals used were of analytical and molecular grade. NG108-15 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2% penicillin/streptomycin, 1% amphotericin B, and HAT as a complete medium. Cells were cultured at 37 °C in 5% CO_2 atmosphere with 95% humidity and checked routinely under inverted microscope (Motic) for any contamination. NG108-15 cell viability was assessed by MTT assay[107]. Metabolically active cells containing mitochondrial dehydrogenase convert the tetrazolium salt, MTT, into insoluble purple formazan crystals at a rate that is proportional to the cell viability. NG108-15 cells were rinsed with PBS, harvested with accutase and plated at a total density of 5 x 10^3 cells/well in a 96-well plate and left to adhere for 48 hours. Cells were preincubated for 2 hours with compounds prior to H_2O_2 (2 mM) exposure for

subsequent 10 hours. 20 µL MTT solution (5 mg/mL) (Sigma Aldrich) was added and incubated at 37°C for another 4 hours. The medium was removed subsequently and DMSO was added to dissolve the formazan crystals. The eluted samples were measured directly in a microplate reader (ASYS UVM340) at 570 nm (with a reference wavelength of 650 nm). Cell viability was calculated based on the formula below:

% of cell viability = [absorbance of treated cells (A_s)/ absorbance of control cells (A_C)] x 100%

The neurite outgrowth analysis was conducted based on protocol discussed by Lozano *et al.*[112] and Mitchell *et al.*[113]. The cells were primed by changing the medium to 5% FBS for 4 - 6 days prior to the neurite outgrowth assay. Cells were plated into at cell density of 2×10^4 cells and were left to adhere overnight. Cells were then treated with different range of concentration of compounds at 48 hour in order to determine the best concentration of the compound that would induce neurite outgrowth without toxicity effect. For comparison purpose, cells were also treated with different concentration of NGF-2.5S from murine submaxillary gland (Promega) which served as positive control. Cells with 5% serum medium were used as negative control. Cell bearing neurite was scored positive if it possesses polygonal morphology with a thin neurite extension with length greater than twofold the cell body diameter and possessing a terminal growth cone. Cells were photographed with inverted microscope (Leica Inverted Fluorescence Microscope, DM16000B) using phase-contrast objectives and images of ten fields per well were taken with an average of 100 cells per field and examined. The percentage of neurite-bearing cells were quantified by scoring total number of neurite-bearing cells and total number of viable cells in 10 microscopic fields.

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